Food and Nutrition

Maintaining and Improving Health

Dave Stewart

Food and Nutrition: Maintaining and Improving Health

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Preface

Food and nutrition science is concerned with the study of nutritional value of food and analysis of nutritional contribution in growth, health, reproduction and immunity of a living organism. For the improvement and management of health, it is necessary to consume rich amount of nutrients in the form of a balanced diet. Macronutrients like carbohydrates, fats and proteins provide structural material for cell membranes and also provide energy. Micronutrients such as minerals and vitamins are essential for good health of living organisms. The aim of this book is to present crucial principles and methods relevant to this field in a comprehensive manner while also bringing into focus, their practical ramifications in the field of health management. It brings forth some of the most innovative concepts and elucidates the unexplored aspects of food and nutrition science. Researchers and students in this field will be assisted by this book.

This book has been the outcome of endless efforts put in by authors and researchers on various issues and topics within the field. The book is a comprehensive collection of significant researches that are addressed in a variety of chapters. It will surely enhance the knowledge of the field among readers across the globe.

It gives us an immense pleasure to thank our researchers and authors for their efforts to submit their piece of writing before the deadlines. Finally in the end, I would like to thank my family and colleagues who have been a great source of inspiration and support.

Editor

Degradation of Gluten in Wheat Bran and Bread Drink by Means of a Proline-Specific Peptidase

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Abstract

Gluten-free wheat bran and bread drink were produced by degrading gluten with *Aspergillus niger* prolyl endopeptidase (AN-PEP). For this purpose, bran from native and germinated wheat grains as well as bread drink were mixed with AN-PEP and incubated at 50°C under different conditions. The amount of enzyme activity ($1.2 \cdot 10^{-4}$ - $8.7 \cdot 10^{-1}$ U), the incubation time (0-72 h), and the pH value (1.0-9.0) were systematically altered. The gluten content was monitored by a competitive ELISA using the R5 antibody. Gluten in two wheat bran samples produced in the laboratory was degraded below the threshold for gluten-free foods of 20 mg/kg. This was not possible in a commercial wheat bran sample that had potentially been heat-treated leading to strong crosslinking of gluten by incorporation of gliadins into the glutenin fraction, possible formation of isopeptide crosslinks and, thus, poor digestibility. In contrast, gluten in bread drink was easily degraded after a short incubation time of 30 min and low AN-PEP activity. No significant differences of the quality parameters between treated and untreated products were found. Remarkably, bran from germinated grains was strongly enriched in nutritionally positive compounds such as dietary fiber and folates compared to native grains. Thus, wheat bran from germinated grains rendered gluten-free by treatment with AN-PEP can contribute to increasing the nutritional value of the gluten-free diet.

Keywords: Celiac disease; Gluten degradation; Prolyl endopeptidase; Wheat bran; Bread drink

Introduction

Celiac disease (CD), also called gluten-sensitive enteropathy, is an inflammatory disorder of the upper small intestine, affecting about 1% genetically predisposed individuals of the Western population. Therefore, it is one of the most frequent food intolerances worldwide. Storage proteins from wheat, rye, barley, and possibly oats, which are called gluten in the field of CD, trigger an immune response in the small intestine leading to an inflammation and ultimately to the destruction of the microvilli necessary for the absorption of nutrients. The only treatment is to maintain a strict gluten-free diet with an intake of less than 10 mg of gluten per day [1]. Dietetic gluten-free products are regulated by the Codex Alimentarius Standard as well as the Commission Regulation (EC) No 41/2009. A maximum of 20 mg gluten/kg food is allowed in order to justify a "gluten-free" claim [2,3]. In some countries, products with gluten contents between 20 and 100 mg/kg can be declared as "low in gluten".

Not only are gluten-free products more expensive than their gluten-containing counterparts, they also differ in regard to aroma, taste and texture and, most importantly, nutritional value [4]. Several studies revealed that numerous adult CD patients on a gluten-free diet show signs of poor status of vitamins (folate, B_6, B_{12}), minerals (iron, calcium), and fiber [5-7]. A logical approach to improving this situation would be to either render traditional gluten-containing raw materials gluten-free, or to increase the content of bioactive constituents in gluten-free foods by processing or fortification.

The goal of degrading gluten in gluten-containing raw materials without altering their quality can be achieved by using so-called prolyl endopeptidases (PEP) (reviewed by [8]). Unlike gastrointestinal human peptidases, these enzymes cleave peptide bonds next to proline residues, which are frequently occurring in gluten proteins, and are able to degrade gluten to CD-inactive peptides containing less than nine amino acids. Possible sources for PEP are bacteria [9], fungi [10], and germinated cereal grains [11]. Previous studies have shown that AN-PEP, an *Aspergillus niger* prolyl endopeptidase, is not only highly active towards celiac-active substrates, but also capable of completely eliminating gluten from wheat starch with contents up to 2,000 mg gluten per kg [12]. AN-PEP was initially developed as 'Brewers Clarex™' for the prevention of chill-haze in beer and the de-bittering of protein hydrolyzates. It can be applied for the production of gluten-free foods from gluten-containing raw materials [13]. A gluten-free beer introduced by the Craft Brew Alliance (Portland, OR, USA) in 2012, is brewed using traditional ingredients such as malted barley. Contained gluten is degraded by adding Brewers Clarex™ during the brewing process [14]. However, the use of AN-PEP to 'deglutenize' further foods has not been described up to now.

Bran is a food ingredient with a high content of bioactive compounds such as dietary fiber, minerals, and folic acid. As part of gluten-free foods it would, therefore, have the potential to improve the nutritional status of CD patients on a gluten-free diet. Furthermore, bran is free of cholesterol and comparatively low in calories. Bran from germinated cereals would even be more suitable for gluten-free foods, because germination of cereals under controlled conditions not only induces peptidase activity supporting gluten-degradation, but also increases the contents of valuable nutrients [15].

Beverages obtained by fermentation of cereals or cereal products have gained considerable popularity. This is not only true for beer, but also for cereal-based soft drinks. Bread drink originates from Russia

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('Kwas') and is produced by subjecting sourdough bread from wheat, rye, or oats to a non-alcoholic lactic acid fermentation for up to six months [16]. Bread drink is mainly consumed because of its health-promoting effects [17,18] and has reached a considerable sales volume. However, the product contains gluten in a concentration above 20 mg/kg and can, therefore, not be consumed by CD patients. Based on previous work, it should be possible to remove residual gluten in bread drink to enable a gluten-free claim.

Therefore, the aim of the present study was to produce gluten-free wheat bran and bread drink by using AN-PEP for complete gluten degradation. Furthermore, the content of bioactive compounds such as folate and dietary fiber in bran should be increased by germination of wheat grain. This type of food would contribute to increasing the nutritional value of the gluten-free diet.

Material and Methods

Chemicals

All chemicals were purchased from Sigma Aldrich (Steinheim, Germany) or VWR (Darmstadt, Germany) at analytical or higher grade.

Food samples

Common wheat cv. Hermann 2011 (bran 1) and germinated common wheat cv. Hermann 2009 (bran 2, germination for 7 days at 25°C as described in [19]) were milled on a Quadrumat Junior mill (Brabender, Duisburg, Germany) and sieved yielding white flour (particle size <0.2 mm) and bran (>0.2 mm). Thus, bran 1 was from non-germinated and bran 2 from germinated wheat. Bran 3 was a commercial sample purchased in a local store. Bread drink from a German producer was used as additional sample.

AN-PEP

A commercially available proline-specific peptidase preparation from *Aspergillus niger* (AN-PEP; DSM Food Specialties B.V., Delft, The Netherlands) was used for the degradation of gluten. The lyophilized fermentation broth of AN-PEP was dissolved in distilled water to obtain different concentrations (0.1 - 750 mg/mL). The gluten-specific peptidase activity was determined according to Schwalb et al. [19]. For comparability to previous work [12], all activity values are based on gliadin as a substrate and pH 4.0. Briefly, gliadin (50 mg) from flour of common wheat cv. Cubus [20] was dissolved in 60 % (v/v) ethanol (20 mL), incubated for 2.5 h at 50°C and filtered through a 0.45 mm membrane. Gliadin solution (130 µL) and peptidase solution (380 µL) were mixed including addition of a buffer to yield a final pH of 4.0 and magnetically stirred for 150 min at 50°C. Incubated samples were analyzed by reversed-phase high-performance liquid chromatography (RP-HPLC) and gliadin degradation was monitored at 210 nm [19]. The specific peptidase activity of AN-PEP based on gliadin as a substrate was 23 U/g [12].

Gluten Degradation by AN-PEP

Wheat bran

Wheat bran samples (5 g) were mixed with AN-PEP solution (50 µL; 1 - 750 mg/mL; $1.2 \bullet 10^{-3}$ – $8.7 \bullet 10^{-1}$ U) and distilled water (20 mL). The pH value was adjusted between 1.0 and 9.0 in increments of 1.0 by adding buffer (1 mL, 0.2 mol/L; pH 1, glycine-HCl; pH 2-4, sodium acetate-acetic acid; pH 5-8, phosphate; pH 9.0, tris(hydroxymethyl) aminomethane/HCl). Vessels were closed, shaken, inserted into a water bath, incubated under continuous agitation, centrifuged (room temperature RT ≈ 20°C, 20 min, 3,760 *g*), and the residue lyophilized. The conditions were modified as follows: Incubation time, 4 - 72 h; temperature, 4, 20, 30, 40, 50, 60°C at pH 4.0, and 50°C at pH 1 - 9.

Bread drink

Bread drink (5 mL) was mixed with aqueous AN-PEP solution (50 µL; 0.1 - 10 mg/mL; $1.2 \bullet 10^{-4}$ – $1.2 \bullet 10^{-2}$ U), the mixture was vigorously shaken; the vessel was closed, and incubated in a water bath. The reaction was stopped by heating to 90°C for 10 min. The conditions were modified as follows: Incubation time, 0 - 30 min; temperature, 4, 20, 30, 40, 50, 60, 70, 80, 90°C; pH-value, 1 - 9 adjusted with buffer (1 mL) as described above.

Gluten Quantitation by ELISA

Gluten was quantitated by means of a competitive ELISA using the R5 antibody [21] (RIDASCREEN® Gliadin competitive, R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions [22]. Gliadin concentrations were converted into gluten concentrations by multiplying by a factor of two [2,22].

Protein Content and Protein Distribution of Wheat Bran

The crude protein content (N x 5.7) of wheat bran was determined by means of the Dumas method according to ICC standard 167 [23]. A TruSpec Nitrogen Analyzer (Leco, Kirchheim, Germany) was used. The quantitative distribution of Osborne fractions in bran was determined by an extraction/RP-HPLC method according to Wieser et al. [24]. Aliquots of the albumin/globulin, gliadin, and glutenin fractions were analyzed on a Thermo instrument (Thermo Electron Corp., Dreieich, Germany) using a C_{18} silica gel column (2.1 x 150 mm, 3 µm, 30 nm; Dionex, Idstein, Germany) at 60°C. Elution solvent A was 0.1% (v/v) trifluoroacetic acid (TFA), and solvent B was 0.1% (v/v) TFA in acetonitrile. The injection volumes were 15 µL for the albumin/globulin, 15 µL for the prolamin, and 20 µL for glutelin fraction. The solvent gradient was stepwise linear starting with 0-1 min 0% B, 1-11 min 0 - 20% B and 11-14 min 20 - 90% B (albumins/globulins) or 1-17 min 0-30% B and 17-23 min 30-90% B (prolamins, glutelins). The flow rate was 0.3 mL/min and the detection wavelength was 210 nm. Reference gliadin from the Prolamin Working Group (PWG-gliadin) [25] dissolved in 60% (v/v) aqueous ethanol (2.5 mg/mL) was used for calibration.

Dietary Fiber Content of Wheat Bran

The soluble, insoluble, and total dietary fiber content of untreated and AN-PEP treated wheat bran was determined according to AOAC Official Method 991.43 [26]. All samples were used in a homogenous state and measured in duplicates. A total dietary fiber assay kit (Sigma Aldrich, Steinheim, Germany) was used for digestion with α-amylase, protease, and amyloglycosidase.

Folate Content of Wheat Bran

The folate content of untreated and AN-PEP treated wheat bran was determined by means of the VitaFast® Folic Acid Kit (R-Biopharm, Darmstadt, Germany) according to the manufacturer's instructions [27]. Homogenized sample (1 g) was digested with 10 mg chicken pancreatin in the dark at 37°C overnight (16 h) to degrade folate polyglutamates to mono- or diglutamates. Together with a folic acid medium (150 µL), digests (150 µL in different dilutions) were pipetted

into a microtiter plate coated with *Lactobacillus rhamnosus*. The growth of the microorganism depends on the supply of folic acid present in a standard or in the sample solution. Microbial growth was measured as turbidity in a microtiter plate reader (ASYS Expert 96, Biochrom Asys, Cambridge, United Kingdom) at 620 nm and compared to a standard curve with defined folate concentrations (0.16 – 1.28 µg/100 g).

Sensory Analysis of Bread Drink

Untreated and AN-PEP treated bread drink was mixed with apple juice and water (1/1/1, v/v/v) before sensory analysis according to the manufacturer's recommendation for consumption. A triangle test for similarity according to ISO 4120:2004 [28] was carried out to determine whether a perceivable sensory difference existed between the mixes containing treated and untreated bread drink. Conventional sensory profiling was carried out according to ISO 13299:2003 [29] by comparing the bread drink mix for the attributes pleasantness of aroma, taste and aftertaste as well as the intensities of the attributes bitter, acidic, and sweet on a scale from 1 to 5. All sensory experiments were carried out by a panel of 24 persons.

Statistical Analysis

Data was statistically evaluated using Microsoft Office Excel 2010 (Microsoft Corporation, Seattle, Washington, USA). The following values were applied for assessing levels of significance: $p>0.05$, insignificant; $p<0.05$, statistically significant; $p<0.01$, significant; $p<0.001$, highly significant.

Results and Discussion

Gluten degradation in wheat bran

ELISA analysis gave high gluten contents of 107,285 mg/kg, 5,335 mg/kg, and 53,333 mg/kg in bran 1, 2, and 3, respectively. The lower gluten content of bran 2 compared to the other bran samples was a result of germination, which induced endogenous peptidase activity [11] that partially hydrolyzed the gluten to about 5% to 10% of the content present in non-germinated bran. Bran is rich in minerals and folic acid as well as dietary fiber and, therefore, improves the nutritional status of foods. Conventional bran products, mostly produced from wheat or rye are often used to enrich products in dietary fiber in order to guarantee an optimal supply. Our investigations have shown that commercially available bran contains approximately 50,000 mg gluten/kg; the daily recommended consumption of at least 25 g bran [30] would thus add up to a gluten intake of 1,500 mg, which is by far more than can be tolerated by CD patients.

The treatment of bran with AN-PEP at pH 4.0 and 50°C led to a decrease of the gluten content of all samples (Table 1). The following data is based on an amount of 5 g bran. After incubation with AN-PEP (0.12 U) for 48 h, the gluten content of bran 1 was below 20 mg/kg. The same result was obtained after treatment of bran 2 with AN-PEP (0.46 U) for 24 h. The gluten content of bran 2 was even reduced below the limit of quantitation (LOQ; 10 mg gluten/kg) of the ELISA method after a longer incubation time (48 h) with a lower enzyme concentration (0.12 U). The reason was the presence of a low initial gluten concentration due to germination of the grains before milling and bran isolation (see above). Studies on gluten degradation as affected by pH and temperature showed that brans 1 and 2 were obtained gluten-free after incubation at 40-50°C and pH values between 1.0 and 4.0 or without addition of buffers (data not shown).

Unlike the first two bran samples, bran 3 was more resistant to complete gluten degradation. It was not possible to reduce the gluten content of bran 3 to less than 142 mg/kg even with a very high AN-PEP activity (0.87 U) and a long incubation time (72 h) (Table 1). According to the manufacturer's information this bran had not been treated in any specific way and was merely stored under a protective atmosphere to ensure a long shelf life despite of a fat content of 5.0 g/100 g. An Osborne fractionation of all bran samples was performed in order to see if the protein composition was considerably different (Table 2). As compared to bran 1 from minimally processed (= milled) wheat grain (glutenin content 21%), bran 3 had a considerably higher content of glutenins (36%). This indicates some kind of treatment such as excessive heating. Heat treatment can lead to incorporation of gliadins into the glutenin fraction [31,32] by thiol-disulfide interchange. In addition, heating has been shown to induce the formation of isopeptide crosslinks in glutenin [33]. Both disulfide and isopeptide crosslinks would lead to a very compact glutenin structure, which is likely to be more resistant to proteolytic cleavage compared to unmodified glutenin. This would explain the resistance of bran 3 to complete gluten degradation. Altogether, gluten degradation in wheat bran was more difficult than in wheat starch [12] due to its high initial gluten content. For example, wheat starch samples can be deglutenized at temperatures between 4 and 60°C and pH values between 1.0 and 6.0 [12], while gluten degradation in bran (bran 1 and 2) is only possible at 40 to 50°C and pH values between 1.0 and 4.0 or without addition of any buffers.

Influence of AN-PEP treatment on quality parameters of wheat bran

The crude protein content of wheat bran samples before and after AN-PEP treatment showed no significant difference (Table 3). The content of dietary fiber was only weakly affected by the treatment with AN-PEP (Table 4). The total dietary fiber content of bran 2 (untreated, 40.6%; treated, 42.2%) was considerably higher than of

AN-PEP concentration (mg/mL)	Enzyme activity ($U \cdot 10^{-1}$)	Incubation time (h)	Gluten content (mg/kg)[a]		
			Bran 1	Bran 2	Bran 3
100	1.2	24	579 ± 42^{A}	22 ± 2^{A}	904 ± 144^{A}
400	4.6	24	127 ± 3^{B}	16 ± 9^{A}	411 ± 28^{B}
100	1.2	48	15 ± 3^{C}	<LOQ[b]	215 ± 64^{C}
400	4.6	48	5 ± 0^{D}	<LOQ[b]	142 ± 3^{D}
500	5.8	72	n.d.[c]	n.d.[c]	152 ± 29^{CD}
750	8.7	72	n.d.[c]	n.d.[c]	170 ± 9^{C}

[a]Mean value of duplicate determinations ± standard deviation. Values associated with different capital letters within the same sample denote significant differences (two-sided t-test, $p<0.05$)

[b]Limit of quantitation (10 mg gluten/kg)

[c]Not determined

Table 1: Gluten content of bran samples as affected by AN-PEP concentration and incubation time. 5 g bran was incubated with 50 µL AN-PEP solution and incubated at 50 °C and pH 4.0. Initial gluten contents of bran 1, 2, and 3 were 107,285 mg/kg, 5,335 mg/kg, and 53,333 mg/kg.

Sample	Albumins / Globulins (%)[a]	Gliadins (%)[a]	Glutenins (%)[a]
Bran 1	31.3 ± 1.1^{A}	47.5 ± 0.5^{A}	21.2 ± 0.5^{A}
Bran 2	41.1 ± 0.2^{B}	40.3 ± 0.4^{B}	18.6 ± 0.6^{B}
Bran 3	29.4 ± 3.1^{A}	34.5 ± 0.5^{C}	36.1 ± 0.8^{C}

[a]Mean value of duplicate determinations ± standard deviation. Values associated with different capital letters within the same column denote significant differences (two-sided t-test, $p<0.05$).

Table 2: Content of albumins/globulins, gliadins, and glutenins (% of total extractable protein) of bran samples before treatment with AN-PEP

Sample	Protein content (%)[a]	
	Before treatment	After treatment
Bran 1	13.1 ± 0.1	13.1 ± 0.1
Bran 2	13.9 ± 0.1	13.8 ± 0.3
Bran 3	15.6 ± 0.1	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg.

Table 3: Protein content of bran samples before and after AN-PEP treatment. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Protein contents of bran 1 and bran 2 before and after treatment were not significantly different (two-sided t-test, p>0.05).

Sample	Dietary fiber (%)[a]					
	Before treatment			After treatment		
	Soluble	Insoluble	Total	Soluble	Insoluble	Total
Bran 1	3.3 ± 0.4	27.6 ± 0.2	30.9 ± 0.3	1.5 ± 0.1	24.3 ± 0.5	25.8 ± 0.3
Bran 2	1.9 ± 0.0	38.7 ± 0.9	40.6 ± 0.5	1.7 ± 0.1	40.6 ± 0.1	42.2 ± 0.1
Bran 3	3.1 ± 0.0	49.3 ± 0.5	52.3 ± 0.3	n.d.[b]	n.d.[b]	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg

Table 4: Content of soluble, insoluble, and total dietary fiber in bran samples before and after treatment with AN-PEP. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Values for insoluble and total dietary fiber were significantly higher for bran 2 compared to bran 1 (same raw material; two-sided t-test, p>0.05).

Sample	Folate content (µg/kg)[a]	
	Before treatment	After treatment
Bran 1	196 ± 21	266 ± 29
Bran 2	4534 ± 132	4233 ± 30
Bran 3	506 ± 25	n.d.[b]

[a]Mean value of duplicate determinations ± standard deviation

[b]Not determined; bran 3 was not analyzed for its protein content after treatment as gluten was not degraded below 20 mg/kg

Table 5: Folate content of bran samples before and after treatment with AN-PEP. 5 g bran was incubated with 50 µL AN-PEP solution (bran 1, 100 mg/mL, 1.2 • 10^{-1} U, 48 h; bran 2, 400 mg/mL, 4.6 • 10^{-1} U, 24 h; bran 3, 400 mg/mL, 4.6 • 10^{-1} U, 48 h) at 50 °C and pH 4.0. Folate contents of bran 2 were significantly higher than contents of bran 1 and bran 3 (t-test, p<0.05).

bran 1 (untreated, 30.9%; treated, 25.8%). Bran 2 was obtained from germinated wheat grains. Previous work has shown that the dietary fiber content increases during germination [15], and this has been confirmed here. This is important because several studies showed that the intake of dietary fiber is insufficient, in particular among celiac patients [6,7]. This may lead to a compromised bowel function and constipation [7]. Thus, using deglutenized bran from germinated wheat grains is likely to have an additional health benefit for CD patients compared to the use of conventionally produced gluten-free wheat bran.

The total folate content of bran was analyzed by means of a VitaFast® Folic Acid Kit, a microbiological method for the quantitative determination of folic acid in accordance with international norms. Folate concentrations of bran samples before and after treatment ranged from 196 µg/kg (bran 1 before treatment) up to 4233 µg/kg (bran 2 after treatment) (Table 5). No considerable differences between treated and untreated bran samples were observed. Thus, treatment of bran with AN-PEP had no negative effects on folate, and it was obvious that bran 2 from germinated wheat was enriched in folate compared to bran 1 and 3. In summary, germination had two positive effects in this work. On the one hand it decreased the initial gluten content of bran before the addition of AN-PEP, and on the other hand it induced folate biosynthesis in the developing seed resulting in an almost 20-fold increased folate concentration compared to bran 1. This confirms previous studies on germination of wheat, in which a strong increase of different folate vitamers as well as of total folate was found in germinated wheat grain [15].

In summary, the studies on bran have shown that it is possible to render wheat bran gluten-free by peptidase treatment, even when very high initial gluten concentrations are present. An additional health effect can be obtained in the processed bran if the grains were germinated before bran isolation. This leads to an improvement of the nutritional value due to increased folate and fiber, so that gluten-free bran produced in this way can play an important role in the diet of CD patients.

Gluten degradation in bread drink

The initial bread drink had a gluten content of 82.5 mg/kg, which is in the range of many cereal-based beverages [34]. As shown in Figure 1, gluten was easily degraded in this type of food. While an AN-PEP activity of 1.2 • 10^{-4} U only led to a small reduction of the gluten content (5 g sample, 50°C, 0 - 30 min) a tenfold increase of the activity (1.2 • 10^{-3} U) decreased the gluten content to the threshold after an incubation time of 10 min and below the threshold after 30 min. A further increase of the activity by a factor of 10 (1.2 • 10^{-2} U) caused an immediate reduction of the gluten content below 20 mg/kg and a reduction below the LOQ after 10 min. After a 10 min treatment with 1.2 • 10^{-2} U, gluten was reduced under the LOQ in a very broad range of temperatures between 4 and 90°C and pH values between 1.0 and 9.0. Compared to gluten degradation in wheat bran only about 1/100 of the AN-PEP activity was required for bread drink. Furthermore, bread drink could be rendered gluten-free instantly, while at least 24 h were required in wheat bran. This difference is mainly due to (1) the lower initial gluten content of bread drink compared to wheat bran and (2) the presence of largely hydrolyzed gluten in bread drink. Compared to intact gluten, the mean molecular mass of gluten fragments in bread drink is much lower, thus requiring less peptide bonds to be cleaved by the peptidase to achieve peptides consisting of less than nine amino acids, which are no longer toxic to CD patients [35].

Sensory properties of bread drink

The manufacturer of the bread drink recommended consuming

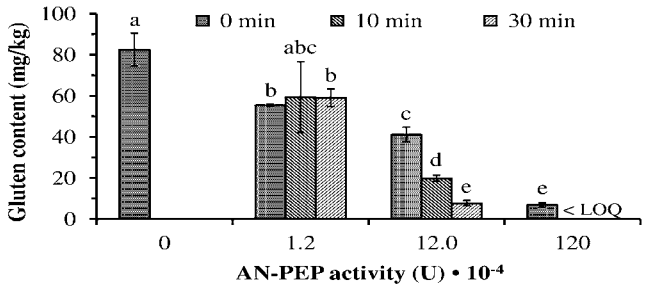

Figure 1: Gluten content (mg/kg) of bread drink as affected by AN-PEP concentration and incubation time. 5 mL bread drink were mixed with 50 µL AN-PEP solution (0.1, 1.0, 10.0 mg/mL, corresponding to 1.2 • 10^{-4}, 12.0 • 10^{-4}, and 120 • 10^{-4} U) and incubated at pH 4.0 and 50 °C for 0 to 30 min. LOQ, limit of quantitation. Values associated with different letters denote significant differences (two-sided t-test, p<0.05).

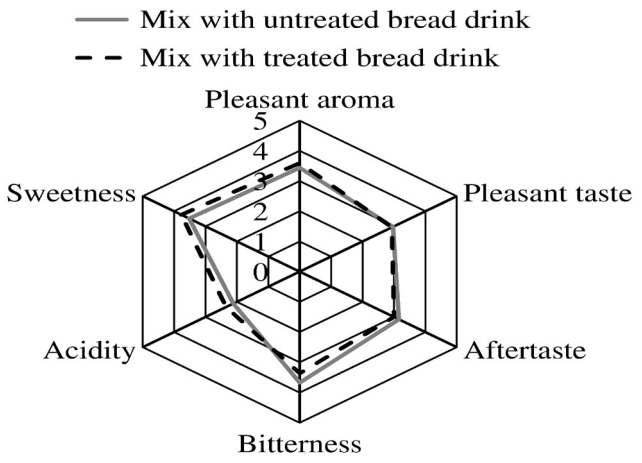

Figure 2: Sensory profiles of bread drink mixes (bread drink/apple juice/water, 1/1/1, v/v/v) containing untreated and gluten-free bread drink. 5 mL bread drink were mixed with 50 µL AN-PEP solution (10 mg/mL, corresponding to 1.2 • 10^{-2} U) and incubated at RT for 10 min. Profiles are not significantly different (p>0.05 for all attributes).

it as mixture of equal volumes of bread drink, apple juice, and water. Therefore, sensory evaluation was carried out with this mixture. A triangle test for similarity was carried out with a sensory panel of 24 individuals to detect an existing difference or similarity between the treated and untreated bread drink. This method is statistically more efficient than a duo-trio test and can be used to detect whether a difference exists in a single or in several sensory attributes. In the first part of the experiment one of the three samples was gluten-free, while one gluten-containing sample was present in the second part. Nine out of 24 panelists properly recognized the different sample. Statistical evaluation gave a type two error of β=0.10 (the likelihood of a conclusion that no distinguishable difference exists, although there is a difference). This means that less than 30% of the population mean can detect a difference between the untreated and the AN-PEP treated bread drink with a confidence level of 90%, i.e. only slight differences were present.

Conventional sensory profiling of the bread drink mix was carried out in addition to the triangle test. The panelists had to evaluate both samples separately for different attributes. Aroma, taste and aftertaste were assessed for their pleasantness, while the intensities of acidity, bitterness and sweetness had to be rated, each on a scale from 1 to 5. The results are shown in Figure 2. Although the mix containing the gluten-free bread drink was evaluated slightly more acidic and marginally less bitter than the gluten-containing mix, profiles were not significantly different (all statistical significances >0.05: pleasant aroma, 0.6; pleasant taste, 0.9; aftertaste, 0.6; bitterness, 0.5; acidity, 0.4; sweetness, 0.5). Therefore, it can be concluded that treatment of bread drink with AN-PEP for gluten degradation is possible without any adverse effects regarding its taste and aroma.

Conclusions

This study is an example that gluten-containing foods can be rendered gluten-free by enzymatic hydrolysis of gluten and gluten fragments. AN-PEP appears to be the enzyme of choice because it manages to degrade even very high gluten concentrations (≈100,000 mg/kg) in different matrices at pH values between 1.0 and 4.0 and temperatures between 40 and 50°C without any adverse effects. Strains of the genus *Aspergillus* have a food grade status, therefore, AN-PEP is suitable to be used in the production of food. The nutritional value of wheat bran can be increased by germination. In combination with enzymatic gluten degradation, products with high contents of dietary fiber and folates can be obtained that are interesting ingredients for healthy gluten-free foods. Bread drink can easily be rendered gluten-free by peptidase treatment without impacting its taste and aroma. This increases the choice of gluten-free foods for CD patients.

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Physico-Chemical Properties of Palmyrah fruit Pulp (*Borassus flabellifer* L)

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Abstract

Palmyrah fruit pulp is rich in nutritional values there is high scope for squash and jam preparation. The estimated trees in India are about 122 millions in which Andhra Pradesh itself having 50 millions. A number value added products from palmyrah available in market, but the fruit pulp is not commonly used for preservation and development of products due to lack of basic information of physico-chemical properties. Hence the study was taken up to analyse the pulp for physicochemical properties for development of health foods. Form this study it was observed that the fresh pulp powder contains, the moisture content was 74.5%. The ash and fat contents (wet matter basis) were 1.2% and 0.8% respectively. The protein content and carbohydrate content were 1.25% and 22.5% respectively. The caloric value obtained was 102.83 kcal/100 g. The pH value was 5.5. Water absorption capacity (18%) and bulk density (0.78 g/cm^3) were recorded for the palmyrah pulp powder. The values for swelling power and foam capacity were 4% and 2.5% respectively. The values gives significant values which can utilized direct or combined with other pulps for preparation of foods.

Keywords: Palmyrah Palm; Fruit pulp; Physic chemical properties

Introduction

The palmyra palm tree is a dioecious plant India stands first in the world in terms of its wealth of palmyrah (*Borassus flabellifer L*) palms with a population nearly 122 million palms [1].

The palm is found growing in Andhra Pradesh, Tamil Nadu, Bihar and Orissa and more number of palms is found in southern states of India. Palmyrah palm has great economic potential and every part of the palm is use full in one way or the other more than 88% of the Palmyrah is used for the welfare of the people, it serves as food (fruit, sap, young shoots) as a building material (the stem, the leaves) It is also used in the pharmacopoeia (roots, male inflorescence) and the leaves are used to make a variety of objects, brooms, baskets, fences and roofs [2] palm wine extracted form palmyrah plays an importation role in the diet.

Fruits mature during august and the ripe fruits fall from the palm during September and October. Each female palm may bear 10-20 bunches of about 200-300 fruits per year. When the fruit is very young, and the top of the fruit is cut off, you find usually three sockets inside and these contain he kernel which is soft as jelly, and translucent like ice, and is accompanied by a watery sweetish liquid. The mature fruit is usually tossed over low burning fire or embers to cook them mildly and the skin is peeled off to expose the juicy fruit. This is squeezed and the pulp removed. The pulp in itself is sweet and creamy and is delicious to eat. The pulp is usually sucked directly from the fibres of the fruit. The fresh pulp is reportedly rich in vitamins A and C. Palmyrah fruit pulp could be commercially utilized to produce food items and animal feed. The whole fruit contains about 40% of undiluted pulp which is dark yellow in colour with its characteristic flavor and bitterness. The pulp is extracted manually with water. Palmyrah pulp is mixed with other fruits for making jam, cordial, cream etc. since its pulp is bitter in taste, it is better to prepare mixed fruit jam rather than palmyah jam separately. To prepare cordials, citric acid is added to its diluted pulp and boiled. Well boiled cordial is bottled in white or amber colured bottles after adding approved food preservative [2]. Although utilization palmyrah fruit pulp is extensive, the literature on physic chemical properties is very limited. Hence this study was carried out to know the basic information of fruit and fruit pulp for developing value added products.

Material and Methods

The fruits (100 numbers) were collected randomly and taken weights for basic data to know the potential of pulp form palmyrah. The ripen palmyrah fruit pulp was collected and stored at cold room temperature (4°C) some of the pulp was direct at 6°C for 24-48 hrs. The dried pulp was finally milled using pulveriser to pass through a 250 um seave. The samples were then packaged in polyethylene bag (150) and kept in a refrigerator (4°C) until needed for use.

Functional properties

Color: The Color of the fresh pulp is observed visually and % transmission [3].

Solubility: The solubility tests were conducted with water, alcohol and acids.

pH: The pH of the fresh pulp was measured with digital pH meter using standard procedure [3].

TSS (total soluble solids): The T.S.S. of the fresh pulp is measured with hand refractrometer [3].

Water absorption capacity: This was determined using methods described by Beuchat [4] one gram sample was weighed into 25 ml graduated conical centrifuge tubes and about 10 ml of water added.

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The suspensions were allowed to stand at room temperature (30+2°C) for 1 hr. The suspension was centrifuge at 200xg (2000 rpm) for 30 min. The volume of water on the sediment was measured and the water absorbed is expressed as percent water absorption based on the original sample weight.

Bulk density: This was determined by the method of Narayana and Narasinga Rao [5] a graduated cylinder tubes were weighed and pulp powder sample filled to 5 ml by constant tapping until there was no further change in volume. The contents were weighed and the difference in weight determined. The bulk density was computed as grams per milliliter of the sample

Swelling power: This was determined with the method described by Leach et al. [6] with modification for small samples one gram of the sample was mixed with 10 ml distilled water in a centrifuge tube and heated at 80°C for 30 min. The mixture was continually shaken the heating period. After heating, the suspension was centrifuged at 1000 x g for 15 min. The supernatant was decanted and the weight of the paste taken. The swelling power was calculated as swelling power=weight of the paste/weight of dry sample.

Foam capacity and foam stability: The method described by Narayana and Narasinga Rao [5] was used for the determination of foam capacity (FC) and foam stability (FS) Two grams of pulp sample was added to 50 ml distilled water at 30+ 2°C in a 100 ml measuring cylinder. The suspension was mixed and properly shaken to foam and the volume of the foam after 30s was recorded. The FC was expressed as a percentage increase in volume. The foam volume was recorded in 1 hr after whipping to determine the FS as a percentage of the initial foam volume.

Physico-chemical properties

Moisture: The study was done by AOAC methods, about 10 g of the material is weighed (M1) into porcelain crucible and placed in an oven at 100-105°C and collected in a desiccators. The process of heating and cooling is repeated till a constant weight is achieved (M2) the moisture percentage is given by

Moisture (% wet basis)=(M1-M2)/M1 X 100

Protein by lowry method: This method was determined by Lowry et al., the lowry folin-ciocalteu (FC) reagents enables the determination of phenolic groups of tyrosine the fresh pulp sample was extracted with buffer and pipette out 0.1 to 0.2 ml of sample extract and make up to 1 ml with water. Add 5 ml of Alkalin copper and incubate for 10 min then add 0.5 ml of FC regent and incubate for 30 min read the O.D at 660 nm. The working standard proteins were prepared with 200 µg/ml. Draw the standard curve.

Lipid extraction: Lipids were extracted according to the method using soxhlet apparatus [3]. A mass M of each dry sample was weighed and introduced in to a previously weighed wattman cartridge. A cotton swab is then placed on top of the cartridge to prevent the rise during heating soxhlet apparatus and extracted with anhydrous ether for about 16 hr. The ether extract is filtered in to a weighed conical flask (M1) after an extraction time the solvent was evaporated on a rotary evaporator. The flask is dried in a desiccators for 2 hours and then weighed with the fat (M2)

The fact content is given by the equation

$$T=\frac{(M2-M1)\times 100}{M1}$$

Ash: The ash content was determined by AOAC method. About 5 g of pulp powder was calcified in a muffle furnace at 450°C during for 3-6 hr. The residue was weigh and converted to a percentage of ash.

Carbohydrate: the carbohydrate content was calculated by difference method.

I.e=100 - (m.c%+fat%+ protein%+ash%)

Identification of carbohydrates: The qualitative tests were performed to identify the carbohydrates i.e Molish test, Fehcing's test, Bendicts test, Seliwanofii's test, Barfoed's test, Bia's test, Inversion test and osazone test, for identifying the Amino Acids i.e Ninhydrin test, Xanthoprotic test, Millons test, Hopkins cole test, Paulys test, Selivons test, Ehrlichs test, Nitrotrusside tests were performed.

Total sugars: Fresh pulp of 100 mg was hydrolyzeed by keeping it in a boiling water bath for 3 hr with 5 ml of 2.5 n HCL of cool to room temperature. Then neutralized it with solid sodium carbonate and make up the vol to 100 ml of centrifuge. Then collect the supernatant and take 0.5 ml of 1 ml of aliquots for analysis. Prepare the standards by taking '0' as blank and 0.2 to 1 ml of working standard glucose (0.1 mg/ml) make up the volume to 1 ml by adding water. Then add 4 ml of anthrone reagent heat for 8 min in a boiling water bath. Cool rapidly and read the green to dark green colour at 630 mm. Draw the standard graph for standards [7-9].

Reducing sugars using 3,5 DNS method: 100 mg of fresh pulp sample was extracted with 80% alchohol pipette out 0.5 to 3 ml of sample of make upto 3 ml with H_2O. They add 3 ml of DNS and heat for 5 min. The add 1 ml of 40% Rochelle salt solution and cool the tubes and take OD at 540 mm using spectro photometer. The standard curve obtained with 1 mg/ml of glucose is used to determine the concentration of reducing sugars sample [7].

Starch by anthrone method : The fresh pulp sample was extracted with 80% hot ethanol add 5.0 ml of H_2O and 6.5 ml 52% perchloric acid kept at 0°C for 20 min and centrifuge save the supernatant repeat the extraction using fresh perchloric acid. Pipette out 0.1 or 0.2 ml of supernatant and make upto 1 ml with water. The standard glucose sample are prepared with the concentration of 0.1 mg/ml Then add 4 ml of anthrone and heat for 8 min. cool the tubes and take O.D at 630 mm [7].

Non reducing sugars: By subtracting the reducing sugars from the total sugars given the value of Non reducing sugars.

Maltose by 3,5 DNS method: The fresh pulp sample was extracted with 80% warm ethanol and centrifuge collect the supernatant and make up to 2 ml with water. Then add 2 ml of DNS reagent and cover with marble keep in boiling water bath for 10 min. cool and dilate to 10 ml with water and measure the OD at 520 nm. Calculate using standard graph maltose concentration is 1 mg/ml range from 0.1 to 2

Vitamin-C: The pulp sample is extracted with 4% oxalic acid. Then centrifuge pipette out 5 ml of supernatant and 10 ml of 4% oxalic acid and titrate against with the dye i.e. 2,6 dichlorophenol indophenols, observe the pink colour. The working standard prepared with ascorbic acid and titrates against with the dye.

Calcium: Pipette 20 to 100 ml of ash solution into 250 ml beaker add 25 to 50 ml of H_2O if necessary add 10 ml of saturated ammonium oxalate and 2 drops of methyl red indicator. Add dil ammonia and a few drops of acetic acid until the colour is fainst pink. Heat the solution to the boiling point. Allow to stand for overnight or 4 hr at room temperature. Filter through what man No -42 paper wash with

water, till the filtrate is oxalate free. Break the point of the filter paper with platinum wire or pointed glass rod. Washed the precipitate first using hot diluted H_2SO_4 from wash bottle into the beaker in which the calcium was precipitated. Then wash with hot water and titrate while still hot (Temp 70-80°C) with 0.01 u $KMNO_4$ to the first permanent pink colour.

Energy: This determination was made according to the method of Atwater which gives the following heat flow coefficients.

1 g of Carbohydrate Provides - 4 kcal

1 g of fat provides - 9 kcal

1 g of protein provides - 4 kcal

Results and Discussion

Functional properties

The colour of the pulp is light orange colour and gives orange colour wavelength in spectrophotometer. The solubility of the pulp is partially soluble in water, alcohol and acid solutions completely soluble in ether and chloroform. The pH of palmyrah fruit pulp powder is slightly acidic i.e pH range Between 5 to 6 as shown in Table 1. The T.S.S. of the fresh pulp is 16-16.5 Brix, with these desirable characteristics; the pulp can be used for food additive to enrich nutritional values [8].

Water absorption capacity

The water absorption capacity for the Palmyrah pulp powder was 18% (2.5 ml/g) (Table 1). Water absorption capacity describes pulp-water association ability under limited water supply. The result obtained shows that the pulp has a good ability to bind water. This result suggests that Palmyrah pulp powder could be used in bakery industry.

Bulk density

Bulk density is depended upon the particle size of the samples. The value obtained from the study was 0.78 g/cm^3 (Table 1). Bulk density is a measure of heaviness of a pulp sample. It is important for determining packaging requirements, material handling and application in wet processing in the food industry. Since pulps with high bulk densities are used as thickeners in food products, the Palmyrah pulp pulp could be used as a thickener [9].

Swelling power

The result for swelling power is presented in Table 1. The Palmyrah pulp powder has swelling power value of 4. Swelling power is a measure of hydration capacity, because the determination is a weight measure of swollen starch granules and their occluded water. Food eating quality is often connected with retention of water in the swollen starch granules.

Foam capacity (FC)

The foam capacity of the Palmyrah pulp pulp is shown in Table 1. The per cent foam capacity is about 2.5% which is lower in general. Foamability is reported to be related to the amount of solubilized protein [5].

Physico-chemical composition of pulp from palmyrah

The riped fruits data showed that 74% of them were large 3 seeded fruits with weight ranging from 450 g to 2200 g and a mean weight of 950 g of the remaining 18% were two seeded fruits and 8% were single seeded fruits were around 300 g in weight. In this study the average weight of palmyrah a seed was found to be 214 g and the average pulp weight per fruit was about 350 g ripe fruits and their seeds are used on a fairly large and profitable scale.

Moisture

Moisture provides a measure of the water content of the pulp and for that matter its total solid content. It is also an index of storage stability of the pulp. The moisture content of the fresh pulp was 74.77% (Table 1). The lower the moisture content, the better its shelf stability and hence pulp should be dried for storage.

Crude fat

The fat content of the Palmyrah fresh pulp was 0.8% (Table 2). This value is relatively high when compared to other pulps and similar to that reported by Sankaralingam et al. [2].

Crude ash

The per cent ash content of the pulp was 1.20% (Table 1). The ash content is the organic residue remaining after the organic matter has been burnt away. It is not necessarily of exactly the same composition as the mineral matter present in the original pulp as there may be losses due to volatilization or some interactions between constituents.

Crude protein

The per cent crude protein of the pulp was 1.236% (Table 2). The value obtained was however lower than that obtained by Sankaralingam et al. [2]. The difference observed may be contributed by varietal differences, maturation of the seeds and environmental conditions.

Carbohydrate

The major component of the pulp was carbohydrate. The value obtained from the study was 22.5% on fresh pulp and it shows higher carbohydrate content (Table 2).

Parameter	Values
Colour	Light orange
Solubility	Completely soluble in ether and chloroform
PH	5.5 to 6
T.S.S	16.5 Brix
Water absorption capacity (%)	3
Fat absorption capacity (%)	2.8
Bulk density (g/cm3)	0.78
Swelling Power (g/g)	4
Foam capacity (%)	2.5

Table 1: Functional properties of pulp from Palmyrah.

Parameter	Values for 100 g
Moisture	74-77%
Ash	1.2 g
Fat	0.8 g
Total Carbohydrates	22.5 g
Reducing sugar	9.5 g
Non Reducing Sugar	13 g
Starch	12.6 g
Maltose	0.5 g
Protein	1.24 g
Ascorbic acid	16 mg
Calcium	8.76 mg
Energy	102.83 k.cal

Table 2: Physico –chemical composition of pulp from palmyrah.

Energy

The caloric value (energy) of the Palmyrah pulp was 102.8 kcal/100 g (Table 2) on fresh weight basis. Also fresh pulp of 100 g having significant amount of sugars and minerals i.e Reducing sugar is 9.5 g, non-reducing sugar is 13 g, starch is 12.6 g, Maltose is 0.5 g, Ascorbic acid is 16 mg and calcium is 8.76 mg.

Conclusion

Form this study it was observed that the fresh pulp powder contains, the moisture content was 74.5%. The ash and fat contents (wet matter basis) were 1.2% and 0.8% respectively. The protein content and carbohydrate content were 1.25% and 22.5% respectively. The caloric value obtained was 102.83 kcal/100 g. The pH value was 5.5. Water absorption capacity (18%) and bulk density (0.78 g/cm^3) were recorded for the palmyrah pulp powder. The values for swelling power and foam capacity were 4% and 2.5% respectively. The values gives significant values which can utilized direct or combined with other pulps for preparation of foods and has a lot of potential in the food industry, especially its uses as nutritional enrichment in food and food based products. This information may be useful for further studies on anti-microbial and antioxidant activities.

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Genetic Polymorphisms of One-carbon Enzymes Interactively Modify Metabolic Folate Stress and Risks of Hepatocellular Carcinoma Development

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Abstract

Few studies have comprehensively evaluated how genetic modifiers of metabolic one-carbon stress as folate deficiency and hyperhomocysteinemia interact to modify the host-susceptibility of human heaptocellular carcinoma (HCC) development. Genetic polymorphisms of 3 key one-carbon enzymes (methylenetetrahydrofolate reductase: MTHFR, methionine synthase: MS, and thymidylate synthase: TS) at 5 loci were characterized in a case-control and hospital-based Asian population (n=398). The monopolymorphic analysis revealed that the *T* variant allele at *MTHFR 677 loci* in relative to the other genotyped variant alleles (*MTHFR 1298C*, *MS 2756G*, *TSER 2R*, *TS3'UTR 1494+6bp* insertion) was associated with a significant 40% reduction of HCC risks in the dominant model (adjusted ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Among individuals with low metabolic folate stress (serum folate>6 ng/mL), the *MTHFR* CC wild-type interacted with the *TSER* 2R variant alleles to increase HCC risks (OR: 0.14 vs. 0.3; 95% CI: 0.1-0.8), whereas 2-fold reduced HCC were associated with the compound *MTHFR T* and *TSER* 2R variant alleles (OR: 0.14 vs. 0.07; 95% CI: 0.02-0.2) (*P* for interaction: 0.044) after adjustment for serum homocysteine (Hcy) levels. The *TSER 2R* or *TS3'UTR+6bp* variant alleles interacted with *MTHFR T* variant allele to reverse its lowering serum folate and elevating Hcy effects (*P* for trend=0.009 and 0.001, respectively). Taken together, our data demonstrated that *MTHFR 677 T* and *TSER* 2R variant allele interacted to alleviate metabolic one-carbon folate stress, which folate-genetic interactions may be the important elements in favor of reduced HCC risks.

Keywords: Metabolic folate stress; Genetic polymorphisms; Hepatocellular carcinoma

Introduction

Serving as one one-carbon donor or acceptor to mediate *de novo* synthesis of purine and pyrimidine, folate-mediated one-carbon metabolism is critical for normal biochemical and physiological function of the liver [1]. Depleted folate status resulted in one-carbon metabolic and genetic stress including elevated homocysteine (Hcy) levels, aberrant DNA methylation, oxidative DNA, lipid and protein damage in hepatocytes and liver tissues [2,3]. Numerous animal studies have demonstrated that elevated one-carbon metabolic stress by dietary folate deprivation led to hepatocellular carcinoma (HCC) development [3-5]. Despite several hepatic disorders subgroups are reported to frequently suffer from folate deficiency [6-8], there are relatively limited human studies to demonstrate relationships between the increased folate-mediated one-carbon stress and HCC carcinogenesis. A prospective high-risk cohort study showed an association of low blood folate with risks for liver damage and HCC on Caucasian population [9]. Asia subjects in high metabolic one-carbon stress as low serum folate and elevated Hcy levels had significantly increased risks for HCC development [10]. The accumulating evidence suggests an universally important role of elevated one-carbon metabolic stress in human HCC development among different races.

Genetic polymorphisms of one-carbon enzymes have been proposed to modulate metabolic one-carbon stress of human subjects. The most studied key enzyme in folate-mediated one-carbon metabolism is the methylenetetrahydrofolate reductase (MTHFR). It irreversibly converts 5, 10-methylenetetrahydrofolate to 5-methyltetrahydrofolate for the remethylation of Hcy to methionine by methionine synthetase (MS), providing the precursor of S-adenosylmethionine (SAM) for DNA methylation [11]. A polymorphism at position 677 (C677T) of the *MTHFR* gene is associated with 30% and 65% reduced enzyme activity in heterozygous (CT) and homozygous (TT) variants, respectively [12]. Another polymorphism at position A1298C of the *MTHFR* gene is associated with moderately reduced the enzyme activity [13]. Individuals with the *MTHFR 677T* or 1298C variant alleles commonly have reduced plasma folate levels and increased Hcy concentrations [14,15]. Reduced MS activity by genetic polymorphism at A2756G was associated with elevated Hcy levels [16].

Thymidylate synthase (TS) is the other rate-limiting enzyme which catalyzes the conversion of dUMP to dTMP for DNA synthesis and repair. The potentially functional *TS* polymorphisms, a 28-bp tandem repeat in the *TS* 5'-untranslated enhanced region (*TSER*) [17], or a 6-bp deletion/insertion at nucleotide 1494 in the 3'-untranslated region of the *TS* gene (*TS 3'-UTR*), were correlated with alteration of *TS* expression [18]. As the TS competes with the MTHFR for their mutual substrate of 5, 10-methylenetetra-hydrofolate in one-carbon metabolism, reduced

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mRNA expression levels affected by *TSER* 2R/2R variant allele or *TS3'UTR* -6/-6 variant alleles [17,19,20] have been associated with lower plasma folate and elevated Hcy levels [18,20-22]. Although the results of the reports are not conclusive, polymorphisms of these one-carbon enzymes not only promoted metabolic stress but also genetic stress such as altered DNA methylation, uracil misincoporation and oxidative DNA damage [23-26], all of which have been proposed as plausible mechanisms in human HCC development.

The full complement of one-carbon enzymes involved in methyl and homocysteine metabolisms is tissue specific. The liver is the major organ with the most active one-carbon metabolism. Hepatic activities of *MTHFR*, *MS* and *TS* could be modulated by availability of their substrate and coenzymes, genetic polymorphisms, rates of one-carbon flux and diseases conditions. Few studies have evaluated how genetic modifiers of metabolic one-carbon stress, mainly emerging from folate deficiency and hyperhomocysteinemia, may interact to modify the host-susceptibility of human HCC development. Several studies reported an increased risk for HCC associated with *MTHFR TT* variant [27,28], whereas others reported a reduced HCC risk for individuals with *TT* genotype [29,30]. The genetic *MS* and *TS* polymorphisms have been individually linked to several cancer risks including colorectal cancers [31-33], yet with relatively little evidence on HCC development. None of the reported results comprehensively evaluates folate-polymorphic interactions on HCC risks. We test the hypothesis in a case-control and hospital-based study on Asian population in the area where a high HCC incident prevailed. DNAs extracted from peripheral blood monocytes (PBMC) of HCC cases and HCC-free controls (n=398) were genotyped for genetic polymorphisms of 3 key one-carbon enzymes at 5 loci. Serum folate and Hcy levels were assayed for metabolic one-carbon stress. Interactions between one-carbon genetic polymorphisms and metabolic folate stress in modifying risks of HCC development were investigated.

Materials and Methods

Study subjects

The present study was carried out in the period Jan 2005 to Aug. 2009. Patients with HCC were recruited from two Medical Centers, Chi-Mei Hospital (CMH) and National Taiwan University Hospital (NTUH) at the Southern and Northern Taiwan, respectively, into the B vitamin and HCC Cancer Prevention Study. Details of recruiting HCC patients with diagnostic criteria for the presence of HCC have been described elsewhere [34]. Briefly, the presence of HCC were diagnosed by two physicians with the data of alpha-fetoprotein elevation (>400 ng/mL), liver imaging (by B-type ultrasonography, computed tomography, magnetic resonance imaging, or/and angiography), and/or histologic exam. In total, 199 HCC patients participated in the entire study. The controls were selected from Community Healthy Exam, who participated in a healthy screening program at Chi-Mei Hospital. The controls were matched by sex with the HCC patients. Exclusion criteria included viral infection, chronic liver diseases, and alcohol abuse as assessed by a medical history, a complete physical examination, and routine laboratory evaluation. The study protocol was approved by the Joint Ethical Committee of Fu-Jen University, Taiwan National University and Chi-Mei Hospital. All participants have provided written informed consent to participate in the study. The written consent content covered (1) IRB preliminary examine research project; (2) The screened eligible control subjects; (3) The clinical trial participants consent, based on regulations of Taiwan National University and Chi-Mei Hospital IRB or Fu-Jen University Ethics Committee.

Blood biochemical determinations

Within 1 week following the diagnosis of HCC presence and prior to the subsequent treatment of HCC in scheduled consultations, patients donated fasting blood samples. Blood of the controls was collected during the time they received health examination. Peripheral blood samples were taken after a 12 h fasting period, chilled, and transported to the laboratory. Plasma and serum samples were immediately separated upon arrival and were stored at -80°C until further analysis. Lymphocytes were purified from whole blood using standard Ficoll-Hypaque centrifugation, and were used for molecular genetic analysis in the study. Folate and total homocysteine (tHcy) levels were measured in the serum samples using commercially available radioimmunoassay kits (Becton Dickinson, Orangeburg, NY), and by fluorescence polarization immunoassay (Becton Dickinson) on an Abbott 130 AxSYM system (Becton Dickinson), respectively.

Genetic polymorphisms

The *MTHFR C677T*, *A1298C*, and *MSA2756G* polymorphisms were determined by real-time PCR and melting curve analysis using a Light Cycler instrument (LightCycler, Roche Diagnostics, Mannheim, Germany). The *TSER* tandem repeat polymorphism and *TS 3'-UTR* polymorphism was analyzed by PCR-RFLP as previously described [17,20]. The forward and reverse primers, the hybridization probes and the amply products for each genotype were presented in Table 1. Briefly, the reaction buffer consisted of 4 μL of Light Cycler DNA Master Hybridization Probe mix (Taq DNA polymerase, reaction buffer, dNTP mixture, and 10 mM $MgCl_2$), 200 nM probe 1, 400 nM probe 2, 200 nM primers, and 50 ng DNA. After a preincubation of the reaction mixture at 95°C for 10 min, thermo cycling was carried out at 95°C for 5 s, 55°C for 5 s, and 72°C for 5 s for 40 cycles. The quality control of each genotype was performed by direct DNA sequencing of separated amplified DNA fragments by electrophoresis in a 2% agarose

Gene	Amplified product	Primer and hybridization probe sequence (5'→3')
MTHFR C677T		
Forward	166 bp	5'-TGG CAG GTT ACC CCA AAG G-3'
Reverse		5'-TGA TGC CCA TGT CGG TGC-3'
Probe 1		5'-TGA GGC TGA CCT GAA GCA CTT GAA GGA GAA GGT GTC T-FL
Probe 2		5'-LC Red640-CGG GAG CCG ATT TCA TCA T
MTHFR A1298C		
Forward	183 bp	5'- CTT TGG GGG AGC TGA AGG ACT ACT AC -3'
Reverse		5'-CAC TTT GTG ACC ATT CCG GTT TG-3'
Sensor [A]		5'-CTT CAA AGA CAC TTT CTT CAC TGG TC 3'-FL
Anchor		5'-640-CTC CTC CCC CCA CAT CTT CAG CAG-3'
MS A2756G		
Forward	290 bp	5'- TTG CTC ATC TAT GGC TAT CTT GCA-3'
Reverse		5'-GAC ACT GAA GAC CTC TGA TTT GAA CTA-3'
Probe 1		5'-GAA GAT ATT AGA CAG GAC CAT TAT G-FL-3'
Probe 2		5'-640-GTC TCT CAA GGT AAG TGG TAG AAA CAG ATT-3'
TSER		
Forward	238 bp or 210 bp	5'-CGT GGC TCC TGC GTT TCC C-3'
Reverse		5'-GAG CCG GCC ACA GGC AT-3'
TS 3'UTR		
Forward	158 bp or 152 bp	5'-CAA ATC TGA GGG AGC TGA GT-3'
Reverse		5'-CAG ATA AGT GGC AGT ACA GA-3'

Table 1: The primers and hybridization probes sequence of nucleotide for the analysis of one-carbon metabolism genes.

gel at 100 V for 40 min. DNA sequencing was conducted with a Big Dye Terminator Cycle Sequencing Kit (Perkin Elmer, Wellesley, MA, USA) and an ABI 377 automated sequencer (Applied Biosystems, Foster City, CA, USA).

Statistical analysis

Statistical analyses were performed using the Statistical Analysis System (SAS/STAT version 6.12, SAS Institute, Cary, NC). Chi-square test was used to examine differences in the distribution of selected demographic variables and in genotypic frequencies between cases and controls. Laboratory data of continuous variables were compared using student t tests between cases and controls. We calculated the geometric mean of levels of serum folate and Hcy levels within strata of *MTHFR* and *TS* genotypes and case-control status using analysis of covariance. Logistic regression models were used to examine the associations between folate status, *MTHFR*, *MS* and *TS* genotypes, and risk of HCC. The strength of a given parameter associated with HCC was measured by its odds ratio and the corresponding 95% confidence interval and 2-sided P value. Dependent variables such as serum folate and Hcy levels that were not normally distributed were log-transformed. Differences were considered to be statistically significant for *P* values of <0.05.

Results

Characterization of metabolic one-carbon stress among the study subjects

As metabolic one-carbon status of the study subjects was profiled using blood biochemical markers, mean serum folate levels of the control and HCC cases were 11.7 ± 6.8 and 7.9 ± 5.2 ng/mL, respectively (Table 2). HCC cases had significantly lower serum folate and elevated tHcy concentrations as compared to the healthy control (*P*<0.001) (Table 2). Metabolic one-carbon stress of the study subjects were characterized by clinical folate deficiency (serum folate <6 ng/mL) and hyperhomocysteinemia (Hcy levels >13 μM). Rates of marginal folate deficiency (43%) and hyperhomocysteinemia (32%) among HCC cases were 3-fold and 5-fold higher than those of the controls (P <0.0001), respectively. The data suggested an elevated metabolic one-carbon stress among HCC cases.

Genetic modifiers of metabolic one-carbon stress in the study subjects

To genotype the subjects, the melting curve-histograms representing for the genetic polymorphisms of *MTHFR C677T, MTHFR A1298C, and MS A2756G* loci of several HCC patients by use of hybridization probe and real-time PCR analysis were shown in Figure 1. Figure 2 shows the representative gel images for the genetic polymorphism of *TSER* and *TS 3'-UTR* using restriction fragment length polymorphism (RFLP) analysis. The quality control of each genotype was confirmed by direct DNA sequencing of amplified DNA products among the designated HCC patients (Figure 3). Genotypic and allelic frequencies of the *MTHFR, MS,* and *TS* polymorphisms at 5 loci among the controls and HCC patients are summarized in Table 3. The data revealed that distribution of *MTHFR C677T* variants was 25% CT and 12% TT in the controls, and 31% CT and 8% TT in the HCC group. The T allelic frequencies marginally differed between the controls and HCC cases (29% vs. 23%, *P*=0.05). Both frequencies of *MTHFR A1298C* variant allele in the control (20%) and cases (19%), and *MS* 2756G variant allele in the control (10%) and cases (10%) were not significantly different. Allelic frequencies for the *TSER 2R* variant allele in the control (17%) and HCC cases (19%) did not significantly differ. Neither did frequencies of the *TS* 3'UTR polymorphisms for the minor allele +6/+6 bp insertion in the control (23%) and the cases (27%). All of the genotype distributions were in agreement with Hardy-Weinberg Equilibrium in both cases and controls (data not shown).

	Control subjects	HCC patients	P values
Age, years	57.4 ± 9.8	62.9 ± 11.4	<0.0001
Sex, male/female	133/66	138/61	0.67
Serum folate, ng/mL	11.7 ± 6.8	7.9 ± 5.2	<0.0001
Deficiency rate[2], n (%)	27 (14)	86 (43)	<0.0001
Homocysteine, μmol/L	9.6 ± 3.7	12.3 ± 5.8	<0.0001
Hyperhomocysteinemia[3], n (%)	11 (6)	63 (32)	<0.0001

Table 2: Base line and metabolic one-carbon stress of the control and HCC patients[1,2]. [[1]Values are expressed as mean ± SD for continuous variables and proportions (%) for categorical variables. Statistical differences were determined by Wilcoxon test for continuous variables and by χ^2 test for categorical variables; [2]Folate and tHcy values were log transformed for statistical tests; [3]Folate deficiency was defined as the serum folate less than 6 ng/mL. [4]Hyperhomocysteinemia is defined as the serum Hcy levels greater than >13 μmol/L].

Single one-carbon polymorphism in relation to HCC risks

Logistic regression analysis for HCC risks in relation to each variant allele of one-carbon polymorphism were examined (Table 4). In relative to the homozygous wild-type C allele at the *677* loci of the *MTHFR* gene, the compound heterozygous and homozygous variant *T* allele were associated with a significant 40% reduction of HCC risks (dominant model: Adjusted ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Conversely, the compound heterozygous or homozygous wild-type C allele had a non-significant 40% increase of HCC risks (recessive model: Adjusted OR: 1.4, 95% CI: 0.7-2.8, *P*=0.4). Neither in the dominant nor the recessive model, *TS* polymorphism at single loci of *TSER* or *TS3'UTR* were not significantly associated with HCC risks. The *MTHFR A1298C* variant allele or the *MS 2756G* variant allele did not correlate with HCC risks (data not shown). For sum of *MTHFR* and *TSER* 2R variant alleles, individuals with 3 or 4 mutant alleles exhibited a 50% reduction in risk of HCC as compared to those with no mutant allele, yet without achieving a statistical significance.

Interactions between the single one-carbon polymorphism and metabolic folate stress on HCC risks

We exam how single loci of each one-carbon polymorphism may interact with metabolic one-carbon stress to modify HCC risks by stratifying serum folate status into high metabolic one-carbon stress (deficient folate level <6 ng/mL) or low stress (normal folate level >6 ng/mL). The data are shown in Figure 4. For those with high metabolic stress in folate-deficient status, the compound *CT* and *TT* variant genotypes as compared with the CC genotype were significantly associated with 80% reduced risks of HCC (OR: 0.2, 95% CI: 0.1-0.6, *P*<0.05) (Figure 1A). For those carrying the homozygous *MTHFR CC* wild-type allele, low metabolic folate stress (normal serum folate >6 ng/mL) was associated with a 90% decrease of HCC risks (OR: 0.1, 95% CI: 0.06-0.3, *P*<0.05). Low metabolic folate stress in combination with T variant alleles (CT+TT genotype) further reduced HCC risks by 20% as compared with the wild-type CC genotype (OR: 0.08, 95% CI: 0.04-0.2, *P*<0.05) (Figure 1A). Genetic polymorphisms of *TS* gene at both 3' and 5'UTR loci did not modify HCC risks among individuals with high metabolic folate stress (serum folate <6 ng/mL) (Figure 1B and C). Regardless of TSER and TS 3'UTR polymorphisms, individuals with low metabolic folate stress (serum folate >6 ng/mL) had a significant 70-80% reduced HCC risks as compared with those in high metabolic folate stress (Figures 1B and 2C). No significant interactions between

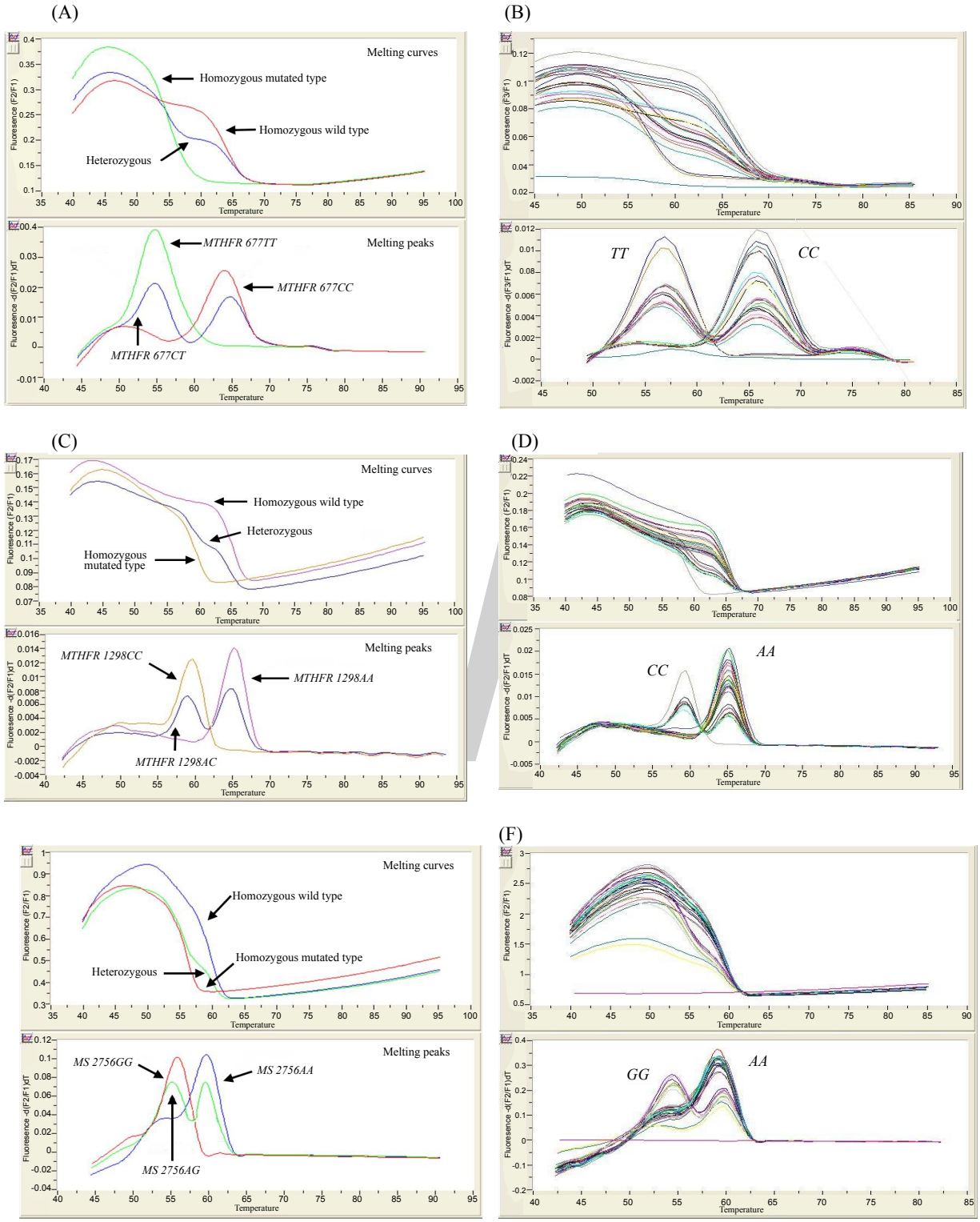

Figure 1: Real-time representative histograms showing genetic polymorphisms at *MTHFR C677T, MTHFR A1298C, and MS A2756G* loci of the subjects. (A) The *MTHFR C677T* genotype was monitored at 640 nm and the melting curve showed a single peak at 64℃ for *CC* samples, a single peak at 55℃ for *TT* samples, and two peaks for heterozygous samples. (B) Genotyping the *MTHFR C677T* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis. (C) The *A1298C* genotype was monitored at 705nm and the melting curve showed a single peak at 63℃ for *AA* samples, a single peak at 60℃ for *CC* samples, and two peaks for heterozygous samples. (D) Genotyping the *MTHFR A1298C* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis. (E) The *MS* A2756G genotype was monitored at 640nm and the melting curve showed a single peak at 60℃ for A/A samples, a single peak at 55℃ for G/G samples, and two peaks for heterozygous samples. (F) Genotyping the MS A2756G *MTHFR* polymorphisms among HCC cases (number C24-28) by use of hybridization probe and real-time PCR analysis.

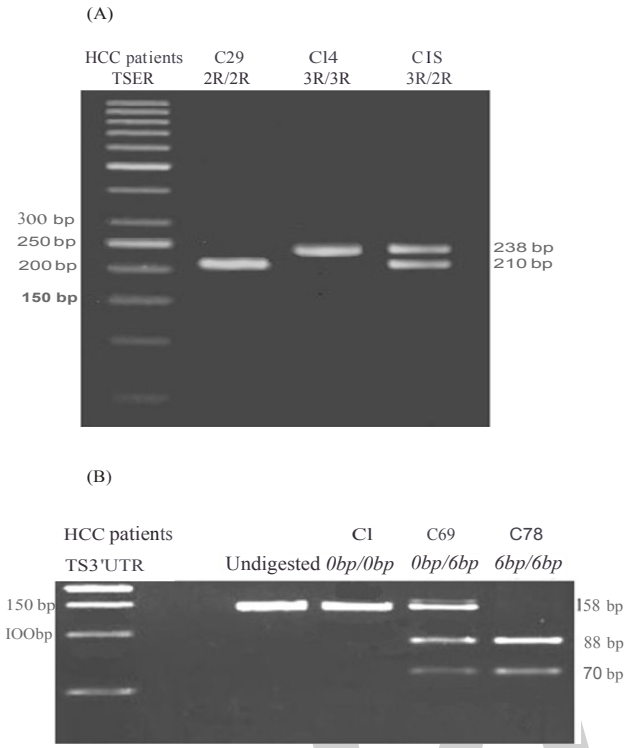

Figure 2: Representative histograms of genetic polymorphism of TSER (A) and TS 3'UTR 1494+6 insertion region (B) using RFLP PCR analysis. (A) Lymphocytic DNAs of the HCC cases (C14, C18 and C29) were assayed for *TSER 3R/3R, 3R/2R, and 2R/2R genotypes.* (B) Lymphocytic DNAs of the HCC cases (C14, C18 and C29) were assayed for *TS 3'UTR 0 bp/0bp*, *0 bp/6 bp insertion, and 6 bp /6 bp.*

single one-carbon polymorphism as *MTHFR C677T, 1298C* variant allele, *MS 2756G* variant allele, TS variant allele, and metabolic folate stress in HCC risks were observed (data not shown).

Interactions between the compound MTHFR and the TS genotype, and the metabolic folate stress on HCC risks.

The interactive effects of the compound *MTHFR* and *TS* polymorphisms and metabolic folate stress on HCC risks were assessed (Table 5). As compared with the compound *MTHFR* 677CC and *TS* 3R/3R wild-types (OR: 0.14, 95% CI: 0.05-0.4), two-fold increased HCC risk was associated with the compound *MTHFR* CC allele and *TSER 2R* variant alleles (OR: 0.3; 95% CI: 0.1-0.8), whereas 2-fold reduced risk was associated with the compound *MTHFR T* variant allele and *TSER* 2R variant allele (OR: 0.07; 95% CI: 0.02-0.2). The folate-polymorphic interactions on HCC risks were significant (*P* for interaction: 0.04). No significant polymorphic interaction on HCC risks was found for the compound *TS3'UTR* genotype and *MTHFR CT/TT* genotypes after adjustment for age, gender, and Hcy levels.

Interactions of the single and the compound TS and MTHFR polymorphisms in modifying metabolic one-carbon stress: serum folate and Hcy levels of the study subjects.

We at last investigate how the *MTHFR* C677T or/and *TSER* variant alleles may interact to modify one-carbon metabolic stress of the study subjects, both of which are risk factors for HCC development (Table 6). Among the control subjects, increased numbers of *MTHFR 677 T* variant alleles in relative to the CC wild-type allele were significantly associated with decreasing serum folate (*P* for trend: 0.004) and elevated Hcy levels (*P* for trend: 0.01). No significant effects of the single *TSER or* the *TS3'UTR* polymorphism on serum folate and tHcy levels of the controls were observed. In relative to the homozygous major allele genotypes for *CC-3R/3R as the* reference, the compound *CT-3R/3R genotypes* were significantly associated with reduced serum folate levels (*P*=0.02). The compound *TT-3R/3R genotypes* was associated

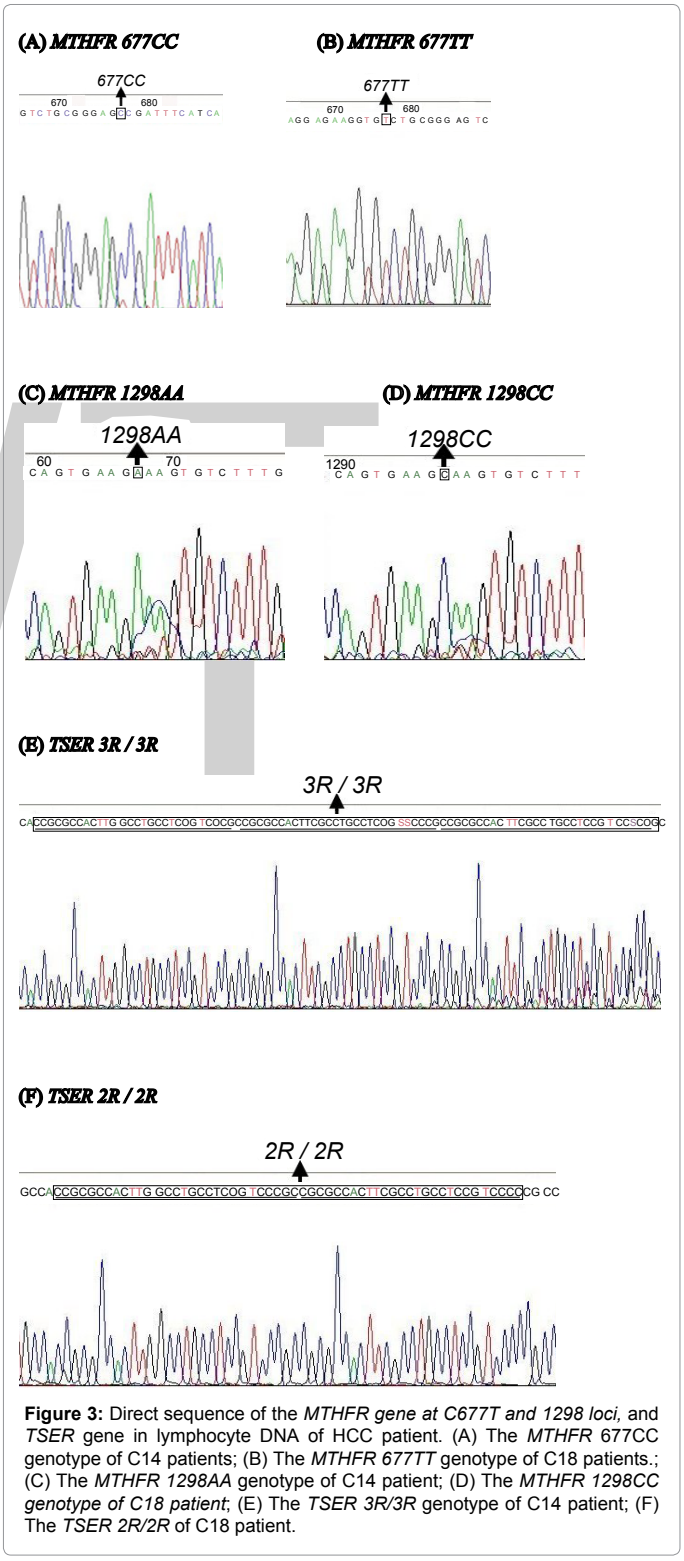

Figure 3: Direct sequence of the *MTHFR gene at C677T and 1298 loci,* and *TSER* gene in lymphocyte DNA of HCC patient. (A) The *MTHFR* 677CC genotype of C14 patients; (B) The *MTHFR 677TT* genotype of C18 patients.; (C) The *MTHFR 1298AA* genotype of C14 patient; (D) The *MTHFR 1298CC genotype of C18 patient*; (E) The *TSER 3R/3R* genotype of C14 patient; (F) The *TSER 2R/2R* of C18 patient.

Genetic polymorphisms	Controls	HCC patients	*P* values
MTHFR C677T, n (%)			
CC	101 (63)	122 (61)	0.11
CT	40 (25)	62 (31)	
TT	19 (12)	15 (8)	
C (%)	71	77	0.05
T (%)	29	23	
MTHFR A1298C, n (%)			
AA	56 (62)	60 (66)	0.53
AC	31 (34)	25 (27)	
CC	3 (3)	5 (5)	
A (%)	79	80	0.84
C (%)	20	19	
MS A2756G, n (%)			
AA	70 (77)	72 (80)	0.72
AG	18 (20)	18 (20)	
GG	2 (2)	0	
A (%)	90	90	1
G (%)	10	10	
TSER, n (%)			
3R/3R	138 (69)	131 (66)	0.71
3R/2R	55 (28)	60 (30)	
2R/2R	6 (3)	8 (4)	
3R (%)	83	81	0.46
2R (%)	17	19	
TS 3'UTR1494, n (%)			
0 bp/0 bp	120 (60)	109 (55)	0.54
0 bp/+6bp	65 (33)	74 (37)	
+6bp/+6bp	14 (7)	16 (8)	
0 bp (%)	77	73	0.29
+6bp (%)	23	27	

Table 3: Genotypic and allelic frequencies of the *MTHFR, MS,* and *TS* polymorphisms at 5 loci among control subjects and patients with hepatocellular carcinoma[1-3] [[1]Abbreviation of the genes: *MTHFR*: Methylenetetrahydrofolate reductase; *MS*: Methionine synthase; TS: Thymidylate synthase; TSER: Enhancer region of TS; TS3'UTR: Un-translate region at 3'end of TS; [2]The 398 subjects were genotyped for *MTHFR C677T*, *TSER* and *TS 3'UTR* polymorphisms. The 180 among 398 subjects were genotyped for *MTHFR A1298C* and *MS A2756G* polymorphisms; [3]Differences in genotypic and allelic frequencies between control subjects and HCC patients were tested by χ^2 test].

with the lowest serum folate levels (P=0.06) and most elevated Hcy levels (P<0.01) as compared with those of the referent. In compound of the variant *T* alleles (CT and TT genotype) with *TSER* 2R variant allele completely negated such *T* allelic effects by normalizing folate and Hcy levels. The compound CC wild-type allele with TSER 2R/2R variant allele, however, reduced serum folate levels as compared with the reference, yet without achieving statistical significance possibly due to the limited subject numbers (n=2).

In relative to the homozygous major allele genotypes of *MTHFR* and *TS3'UTR* for *CC*-0/0bp *as the* reference, the compound *TT*-0/0bp *genotypes* was associated with the lowest serum folate levels (P=0.08) and most elevated Hcy levels (P=0.001). The compound *TT* genotype with heterozygous *TS3'UTR* minor allele genotype (0/+6) negated serum folate lowering effect by T alleles, and in compound with homozygous *TS3'UTR* minor allele genotype (+6/+6) negated Hcy-elevating effects by *T* alleles. Such differentially modifying effects of the *TS* or/and *MTHFR* polymorphisms in serum folate and Hcy levels of the controls were not observed among the HCC cases (data not shown).

Genotypes	Case/Control	OR (95% CI)	P value
*MTHFR C*677T			
CC	223 (122/101)	1.0	-
CT	141 (62/79)	0.7 (0.4~1.0)	0.5
TT	34 (15/19)	0.6 (0.3~1.3)	0.5
P for trend	-	-	0.5
Dominant Model			
CC	223 (122/101)	1.0	-
CT/TT	175 (77/98)	0.6 (0.4~0.9)	0.03
Recessive Model			
TT	34 (15/19)	1.0	-
CC/CT	364 (184/180)	1.4 (0.7~2.8)	0.4
TSER			
3R/3R	269 (131/138)	1.0	-
3R/2R	115 (60/55)	1.2 (0.8~1.9)	0.9
2R/2R	14 (8/6)	1.5 (0.5~4.8)	0.6
P for trend	-	-	0.9
Dominant Model			
3R/3R	269 (131/138)	1.0	-
3R/2R þ 2R/2R	129 (68/61)	1.2 (0.8~1.9)	0.4
Recessive Model			
2R/2R	14 (8/6)	1.0	-
3R/3R + 3R/2R	384 (191/193)	0.7 (0.2~2.2)	0.5
TS3'-UTR			
0/0	229 (109/120)	1.0	-
0/+6bp	139 (74/65)	1.2 (0.8~1.9)	0.7
+6bp/+6bp	30 (16/14)	1.3 (0.6~2.8)	0.7
P for trend	-	-	-
Dominant Model			
0/0	229 (109/120)	1.0	-
0/+6bp + +6bp/+6bp	169 (90/79)	1.2 (0.8~1.9)	0.3
Recessive Model			
+6bp/+6bp	30 (16/14)	1.0	-
0/+6bp + 0/0	368 (183/185)	0.9 (0.4~1.9)	0.7
Sum of *TSER and TS 3'-UTR* variant alleles			
0	187 (91/96)	1.0	-
1	168 (85/83)	1.0 (0.7~1.6)	0.8
2	43 (23/20)	1.2 (0.6~2.5)	0.6
P for trend	-	-	0.8
Sum of *MTHFR* and *TSER* variant alleles			
0-1	310 (160/150)	1.0	-
2	66 (29/37)	0.8 (0.5~1.4)	1.0
3	21 (9/12)	0.5 (0.2~1.4)	1.0
4	1 (1/0)	-	1.0
P for trend	-	-	1.0
Sum of *MTHFR* and *TS 3'-UTR* variant alleles			
0-1	293 (150/143)	1.0	-
2	79 (36/43)	0.8 (0.5~1.3)	1.0
3	23 (12/11)	1.0 (0.4~2.3)	0.7
4	3 (1/2)	0.5 (0.1~5.5)	0.6
P for trend	-	-	0.8

Table 4: Genetic polymorphisms of the methylenetetrahydrofolate reductase (*MTHFR*) and thymidylate synthase (*TS*) genes in relation to risk of hepatocellular carcinoma[1] [[1]Logistical regression analysis with the adjustment for age and sex].

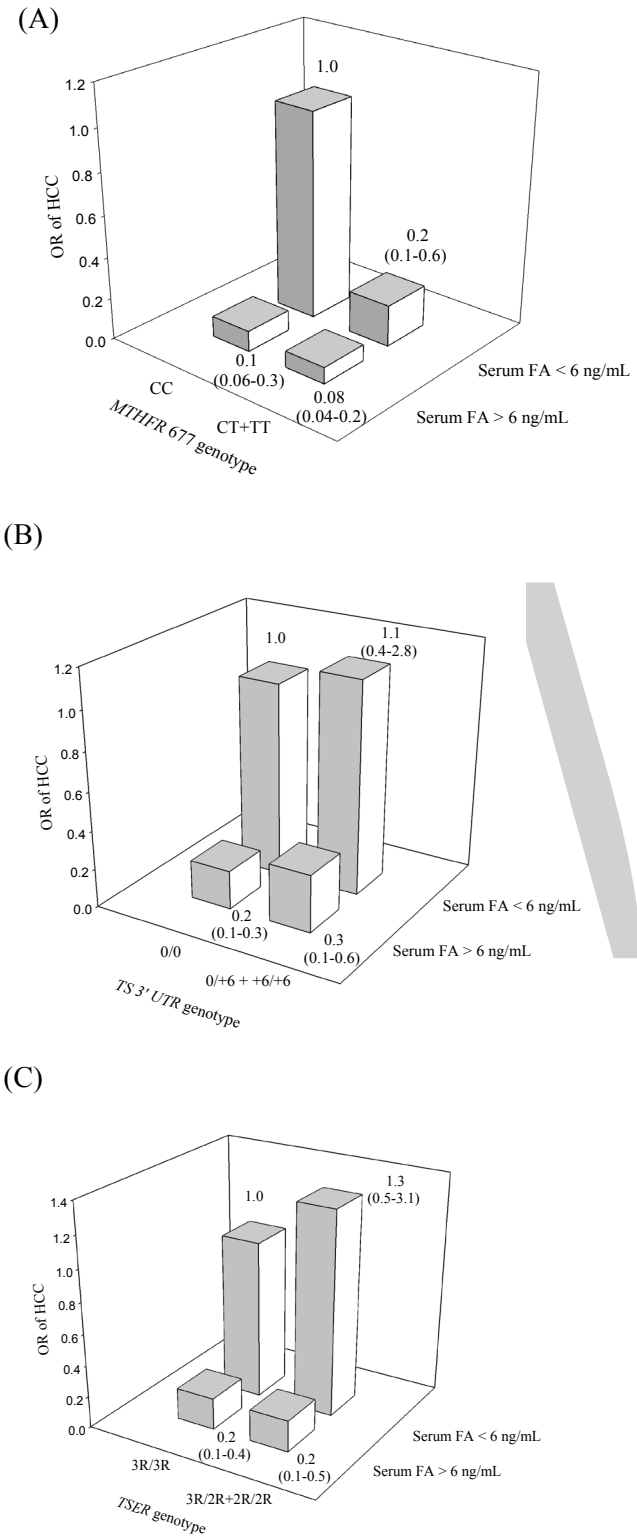

Figure 4: Modifying effects of serum folate levels and genetic polymorphisms on HCC risks. Serum folate levels of the study subjects were stratified into deficient (<6 ng/mL) or normal status (>6 ng/mL), and polymorphic effects on HCC risks on (A) *MTHFR C677T*, (B) *TS* 3'UTR or (C) *TSER* polymorphisms were assessed by logistic regression analysis. HCC risks were adjusted by age, gender and serum Hcy levels [Data were shown by odds ratio (OR) and the corresponding 95% confidence interval (CI) in parenthesis. Statistical significance was set 2-sided P value <0.05 when 95% CI <1.0].

Discussion

Few studies have comprehensively evaluated how genetic modifiers of metabolic one-carbon stress may modify human HCC risks. We found that the *T* variant allele at *MTHFR 677 loci* in relative to the other genotyped variant alleles (*MTHFR 1298C*, *MS 2756G*, *TSER 2R*, *TS3'UTR 1494 +6bp* insertion) was predominantly associated with a significant 40% reduction of HCC risks (ORs: 0.6, 95% CI: 0.4-0.9, *P*=0.03). Based on the mono-polymorphic assessment, several Caucasian population-based studies reported the protective effects of *MTHFR 677TT* alleles against HCC development [29,30,35], whereas the other studies on Asia population did not observe such *T allele*-polymorphic protective effect [27,28]. The possibility for the inconclusive results may in part, if not all, be due to lack of evaluation on the compound polymorphic interactions among the key one-carbon enzymes, corroboration of which activities determined the hepatic one-carbon flow, modulated one-carbon metabolic stress and may interactively modify HCC risks [27-33]. One study by Yuan et al. [30] has demonstrated that the maximum number of mutant alleles in the 3 polymorphic loci of *MTHFR1298, 677, and TS3'UTR* was associated with a significant 62% reduced HCC risks on Caucasians and a non-significant 38% reduced HCC risks on Asians. Similarly, we observed a non-significant 50% reduced HCC risks associated with maximum numbers of variant alleles in the 3 polymorphic loci of *MTHFR677T, TS3'UTR, and TSER* (Table 4). A wide ethnic variation of variant allele frequency reported for *TS* polymorphisms may in part account for the discrepant observations among different races in modifying HCC risks. The *TS 2R variant* allele is less common in Asians (<20%, Table 2) than Caucasians (40%) [36]. The *TS 3'UTR +6/+6* allele was the major allele in Caucasians, whereas this *+6/+6* allele constitutes the minor, and possibly the variant allele genotype in Asians [30,32].

In addition to one-carbon genetic modifiers of HCC risks, metabolic one-carbon stress plays the key role in modulating HCC development in rodents [3-5] and humans [9,10]. The major first-time finding in the present study was to demonstrate that the folate-genetic polymorphisms interacted to modify the host-susceptibility of HCC development. Only among those with low metabolic folate stress, the compound CC genotype and *TSER 2R* variant allele was associated with 2-fold increased HCC risks, whereas the compound *T* and *2R* variant allele was correlated with 2-fold reduced HCC risk (*P* for interaction: 0.044). How folate-genetic interaction may differentially modify HCC risk is unclear. Several mechanisms are plausible. It has been proposed that reduced MTHFR activity by CT and TT genotypes in the absence of other mutations in TS may deviate one carbon flow from remethylation process to de novo thymidylate synthesis, part of which one carbon flow can be redistributed toward purine synthesis with the compound T and 2R variant allele [24]. In particular under normal folate status which provides sufficient one-carbon sources, such one-carbon redistribution by the compound T and 2R variant alleles was reinforced to enrich dNTP pool in favor of DNA repair [25]. Given the fact that the compound *MTHFR T* and *TSER* 2R variant alleles reversed blood folate-lowering and Hcy-elevating effects of TT-allele alone among those with normal blood folate level (Table 6), our observation supported the compound *T* and *2R* variant allelic effects on one-carbon redistribution hypothesis. Enhanced provisions of a better supply of thymidylate and purines by the compound T and 2R variant allele are critical for damaged DNA repair, a defensive mechanism of clearing mutagenic lesions for HCC carcinogenesis [24,31]. It has been reported that the *MTHFR 677TT* genotype was associated with reduced mis-incorporation dU contents of healthy human subjects [23], with reduced DNA mutations of colon tumour [33], and the compound CT/

MTHFR C677T polymorphism	*TS5'-UTR* polymorphism				*TS3'-UTR* polymorphism			
	3R/3R		3R/2R + 2R/2R		0/0		0/+6+6/+6	
	Case/control	OR (95% CI)	Case/control	OR (95% CI)	Case/control	OR (95% CI)	Case/control	OR (95% CI)
Serum folate <6 ng/mL								
677 CC	40 (32/8)	1.0	19 (17/2)	2.3 (0.4~1.3)	36 (30/6)	1.0	24 (20/4)	1.3 (0.3~5.7)
677 CT+TT	25 (16/9)	0.4 (0.1~1.4)	24 (17/7)	0.5 (0.1~1.7)	30 (20/10)	0.4 (0.1~1.3)	20 (13/7)	0.4 (0.1~1.4)
P for interaction	0.54				0.50			
Serum folate ≥ 6 ng/mL								
677 CC	108 (42/66)	0.14 (0.05~0.4)	42 (20/22)	0.3 (0.1~0.8)	85 (32/53)	0.12 (0.04~0.3)	67 (32/35)	0.2 (0.07~0.5)
677 CT+TT	76 (26/50)	0.14 (0.05~0.4)	35 (8/27)	0.07 (0.02~0.2)	64 (18/48)	0.08 (0.03~0.2)	51 (20/31)	0.1 (0.05~0.4)
P for interaction	0.04				0.94			

Table 5: Interactions between MTHFR C677T genotypes, TS genotypes and folate metabolic stress on HCC risk[1, 2] [[1]Logistical regression analysis with the adjustment for age, sex and Hcy levels; [2]Folate and tHcy values were log transformed for statistical tests].

Genotype	Folic acid (ng/mL)				Homocysteine (umol/L)			
	n	Mean	SD	*P*	n	Mean	SD	*P*
MTHFR								
CC	101	14.3	9.7	(referent)	101	9.13	2.3	(referent)
CT	79	12.2	7.9	0.10	79	9.06	2.3	0.56
TT	19	10.0	7.5	0.23	19	11.6	4.5	<0.01
P for trend	-	-	-	0.004	-	-	-	0.01
TSER								
3R/3R	138	12.8	8.2	(referent)	138	9.41	2.8	(referent)
3R/2R	55	14.0	10.4	0.06	55	9.11	2.3	0.16
2R/2R	6	11.8	10.5	0.54	6	10.0	2.3	0.80
P for trend	-	-	-	0.30	-	-	-	0.40
TS3'-UTR								
0/0	120	13.1	9.5	(referent)	120	9.27	2.6	(referent)
0/+6	65	12.9	7.5	0.15	65	9.49	3.1	0.62
+6/+6	14	13.9	10.3	0.77	14	9.30	1.5	0.05
P for trend	-	-	-	0.70	-	-	-	0.70
TSER -MTHFR								
3R/3R-CC	76	14.3	9.2	(referent)	76	9.21	2.3	(referent)
3R/2R-CC	23	14.7	11.6	0.40	23	8.87	2.1	0.78
2R/2R-CC	2	10.8	4.3	0.36	2	9.10	0.8	0.22
3R/3R-CT	51	11.4	6.6	0.02	51	9.09	2.5	0.28
3R/2R-CT	24	13.8	9.6	0.91	24	8.75	1.8	0.15
2R/2R-CT	4	12.3	13.3	0.35	4	10.5	2.8	0.45
3R/3R-TT	11	8.25	4.5	0.06	11	12.2	5.2	<0.01
3R/2R-TT	8	12.4	10.1	0.69	8	10.8	3.4	0.73
2R/2R-TT	0	-	-	-	0	-	-	-
TS3'UTR-MTHFR								
0/0-CC	60	14.5	10.6	(referent)	60	9.11	2.0	(referent)
0/+6-CC	33	15.0	8.6	0.44	33	9.04	2.9	0.15
+6/+6-CC	8	10.1	5.5	0.16	8	9.62	1.2	0.06
0/0-CT	50	12.4	8.4	0.23	50	8.95	2.5	0.10
0/+6-CT	25	10.5	4.5	<0.01	25	9.44	2.0	0.84
+6/+6-CT	4	20.9	13.9	0.52	4	8.05	1.6	0.50
0/0-TT	10	8.00	4.8	0.08	10	11.8	4.1	0.001
0/+6-TT	7	11.3	8.4	0.42	7	11.7	5.8	<0.01
+6/+6-TT	2	15.4	16.4	0.46	2	10.5	1.1	0.23

Table 6: Interactions of the TS or/and MTHFR polymorphisms in modifying metabolic one-carbon stress of the control subjects[1,2] [[1]Values are expressed as mean ± SD for continuous variables. Within each genotype or the compound genotypes, the statistical differences between the referent (wild-type genotype) and the variant genotypes were determined by t test at P<0.05. *P* for trend was analyzed by contrast for linear for continuous variables. Differences were considered to be statistically significant at *P*<0.05; [2]Folate and tHcy values were log transformed for statistical tests].

TT genotypes with reduced oxidative DNA damage of lymphocytes of HCC patients [26]. The reported protection against genetic instability due to the reduced MTHFR activity by T allele may serve as the mechanistic basis to confer host-susceptibility of HCC development [37].

Without being in compound with TT genotype, the 2R/2R double repeat in TSER than the triple repeat alone is associated with lower TS expression in HeLaS3 cells [17] or in tumour tissue [19], which may restrict TS activity to induce deregulation of DNA synthesis, repair and cell cycle progression [18,38,39]. The compound *MTHFR* CC wild-type allele with *TSER* 2R variant allele did not enhance substrate provision of 5, 10-methylene-THF to compensate reduced TS activity. By the observation that the compound *MTHFR* CC wild-type and *TSER* 2R variant alleles displayed an adverse effect in folate status (Table 6) and were associated with 2-fold increased HCC risks (Table 6), our data extended to support the differential folate-genetic interaction in one-carbon folate flow in modifying HCC risk.

It should be noted that the significant *T* and *R* allelic interaction of modulating blood folate status and modifying HCC risks was only observed among those with normal folate status, but not for those with folate-deficiency. Similarly, the compound *MTHFR T* and *TS3'UTR* +6/+6bp variant alleles reversed blood folate-lowering and Hcy-elevating effects of TT-allele alone among those with normal blood folate level (Table 6). As the *TS* 3'UTR +6/+6bp alleles in relative to the 0/0bp alleles were associated with higher expression of TS transcripts [20] and higher levels of serum folate levels [18], the enhanced channeling one-carbon flow toward DNA synthesis and repair by such combined variant genotypes of *MTHFR* and *TS* may favor in recycling one-carbon unit to tetrahydrofolate for reentering the one-carbon metabolism and balancing overall one-carbon flux for each metabolic cycle. As modulation of metabolic one-carbon stress by the compound *MTHFR* and TS variant genotypes is only effective under sufficient folate levels of the control subjects, the data suggest a threashold effect of metabolic one-carbon stress for such folate-polymorphic interactions in the protection against HCC progression. In fact, individuals with low metabolic one-carbon stress (serum folate ≥ 6 ng/L) had significantly 80- 90% reduced HCC risks regardless any examined *MTHFR* and *TS* genotype at any tested loci (Figure 4). The beneficial effects of sufficient folate status in supplying one-carbon units for each metabolic cycle of one-carbon metabolism may maximize the favorable one-carbon flux by altered one-carbon enzymatic activity due to different genetic polymorphisms. Our data also provide the plausible explanation why results of the current literature investigating the *MTHFR* or/and TS polymorphic effects in cancer risks are inconsistent [27-29,32,36]. Without consideration of the preexisting one-carbon folate status with the interactive polymorphic impact as the whole, their real functional associations with cancer risks cannot be truly explicated.

Our findings should be interpreted with a few limitations. Due to the unavailability of tumor specimens, analysis of polymorphic-genetic-folate interactions by germ-line genotypes of MTHFR and TS, and serum folate levels may not reflect the true relationships in the target HCC tissues. Small sample sizes may be underpowered for detecting a small but significant association. Larger sample sizes of the studies are needed to clarify whether the TSER or 3'UTR 1494isert6 polymorphism could truly affect one-carbon folate status in the cases and controls. Lastly, the inherent limitations associated with cross-sectional study designs do not depict the causal effect of *MTHFR* and *TS* polymorphisms, folate status and HCC development. Further prospectively designed studies are warranted.

In summary, our data suggest a strong polymorphic effect by reduced MTHFR activity genotype in serum folate and Hcy levels, both in magnitude levels and functional profiles, was differentially influenced by TS variant allelic interaction in normal folate-dependent threshold levels. The compound polymorphic impact of T and 2R variant allele in reduced HCC risk of folate-sufficient individuals supports the hypothesis that deviation of one carbon flux in favor of thymidylate and purine synthesis, possibly for DNA repair, may be a key anticancer mechanism of HCC carcinogenesis. As TS is a target for chemotherapeutic drugs such as 5-fluorouracil, and its mRNA and protein expression levels as the prognostic indicators for certain cancers [40,41], further studies on effects of polymorphic-folate interaction in HCC survival and prognosis are warranted. Since *MTHFR* and *TS* genetic polymorphisms interacted to modulate metabolic stressor of folate status, one-carbon polymorphic identification should be useful to serve as pre-diagnostic markers of metabolic stress in providing alternative strategies in HCC prevention and prognosis.

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Author Disclosures

Chang-Sheng Kuo and Chin-Pao Cheng contribute equally to the study. CS Kuo, CP Cheng, HT Kuo, CH Chen, CY Huang, CC Chen and RFS Huang: No conflicts of interest.

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Milk Production, Marketing and Processing Practices of Dairy Cattle in Debremarkos Woreda of East Gojjam Zone, Amhara Regional State

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Abstract

Introduction: The study was conducted in Debremarkos Woreda, Amhara National Regional State, from October 2007 to May 2008.

Objectives: The objective of milk production, marketing and processing practices of dairy cattle in debremarkos woreda of east gojjam zone, amhara regional state and to get base line data on the status of dairy cattle production levels in the study area.

Methods: All 7 kebeles of Debremarkos were included in the study. A total of 200 households were selected randomly based on the proportion of total households in each kebele site using random number table. In formations were collected by key informant group discussion, household level questionnaire survey, farm visit and personal observations. The data were analyzed using the SPSS computer software and Excel programs.

Results: The mean land holding in rural farms were 1 ha and except 5 persons the rest of urban area farms were landless. The mean livestock holding was 8.36 TLU in rural areas and 5.32 TLU in urban areas. Cattle constituent 98.86% and 92.44% of total TLU per household in Urban and rural sites respectively. In urban sites cows constitute 41.20% of cattle herd and steers 0.60% and in rural sites steers constitute 20.31% and cows 29.00% of cattle herd. The proportion of cross breed and local breed cows in urban areas was 31.10% and 69.90% and for rural areas it was 3.38% and 96.62% respectively. Main feed resources were crop residues and communal grazing land and most common supplements include hay, atela, wheat bran and noug cake mix as concentrates. For the last two years AI service was not available in the Woreda. During the survey period 72.0% of urban area and 93.2% of rural area households used uncontrolled natural mating. Common animal health problems were black leg, anthrax, abortion, foot and mouth disease, ticks, liver fluke. Animal health service (treatment and vaccination) for the last two years was not available in the Woreda. Only 7.5% of urban area households used private veterinary service. The average daily milk yield was 1.50 ± 0.68 and 7.30 ± 4.65 litters for local and cross bred cows respectively.

Conclusion: There was a significant difference in mean daily milk yield, lactation length, age at first calving and calving interval, for local breed cattle between urban and rural areas but there was no significant difference for cross breeds except calving interval in between urban and rural areas. Main milk processing milk products include butter, ayib, sour milk, butter milk, whey and "Metata." The main milk market outlet was contractual type of informal marketing either for hotels and restaurants or neighboring consumers. Lack of improved breed cattle, disease, feed shortage, milk market, space and water were identified in decreasing order of importance as constraints for dairy production.

Recommendation: From this study, it was recommended that: Extension service should be improved; dairy cooperatives and bull service should be established.

Keywords: Characterization; Dairy cattle; Production systems; Debremarkos

Introduction

Livestock keeping is an ancient tradition of rural areas of Ethiopia. The size and diversity of livestock resources have become vital to sustenance of rural life and in fact the largely agrarian economy of the country. Cattle constitute the predominant element of livestock wealth in Ethiopia both in the agricultural high lands and pastoral and agro-pastoral low lands, and hence the proportional contribution to the national economy is considered to be high. Based on crude assessments, the contribution of cattle to the marketed milk and meat, national wide, is estimated to be 96 and 45%, respectively [1]. Livestock provide food in the form of meat and milk, non-food items such as draft power, manure and transport services as inputs into food crop production, fuel for cooking. Livestock also serve as a source of income through sale of the items, animals, hides and skins. Furthermore they act as a store of wealth and determine social status in the community. Because of these important functions livestock play an important role in improving food security and alleviating poverty.

Although the livestock sector has a significant contribution to the national economy and food self-sufficiency, animal productivity in Ethiopia is extremely low. This is evidenced by the very low per capita consumption of protein and a very low growth rate of milk and meat production [2]. The average milk production capacity of the indigenous cow per head per lactation is estimated at 213 kg which is very low [3]. A survey study showed that average daily milk production per cow was 1.2 L and the average calving interval 27 months [4]. Per capita consumption of milk in Ethiopia is as low as 17 kg per head while the

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average figure for Africa is 26 kg per head. With an annual growth rate of 3.5% the human population in Ethiopia will increase to about 139 million by the year 2020, therefore, the demand for animal products is estimated to increase substantially [3]. To meet the increasing demand for milk and milk products, improvement of the productivity of dairy cattle through appropriate technologies such as breeding programs, intensification of the dairy production systems and development of market infrastructures are crucial steps [5].

The low productivity is due to a number of factors among which are quantitative and qualitative deficiencies in the feed resource base, diseases, poor animal performance level, in adequate livestock policies with respect to extension services, marketing and infrastructure, and insufficient knowledge on the dynamics of the different types of farming systems existing in the country. Among all factors emphasis has been given for the improvement of the genetic potential of the local breeds of cattle in the country. Breed improvement programs for dairy production in Ethiopia were started by importing pure temperate breed of cows during the Italian occupation and since then crossbreeding using temperate breeds with indigenous breeds has been practiced by a number of governmental and non-governmental institutions. However, these efforts have been met with little success because of the various technical, organizational and socioeconomic constraints [6]. The development of genetic improvement programs for cattle will only be successful when accompanied by a good understanding of the production systems and when simultaneously addressing several constrains for example feeding, health control and management [7].

To develop appropriate interventions to assist smallholder dairy households, and identifying those which should be targeted requires a clear understanding of the dairy systems. Characterization is the grouping of farmers with similar practices and circumstances for whom a given recommendation would be broadly appropriate [8]. A study on market-oriented urban and peri- urban dairy production systems in the Addis Ababa milk shed developed by ILRI for general characterization of dairy systems characterized seven, market-oriented, dairy production sub-systems [9].

Four major systems of dairy production can be distinguished in Ethiopia. These are: Lowland pastoral dairy production systems, rural highland small-holder dairy production system, urban and peri-urban small scale dairy production system and large scale dairy production system [10]. The characteristics of dairy production systems in the high lands of Ethiopia are characterized by mixed crop–livestock production system and vary substantially in terms of intensification, management systems, genotypes used, type and methods of marketing and processing of milk and dairy products [6]. Even less is known about the productivity levels, major husbandry constraints and opportunities for realistic improvements in the prevalent production systems [9]. This necessitates the need to characterize the smallholder dairy production systems for livestock improvements based on the level of intensification of the farm dairy system, risk management strategies, level of access to output markets and input services, and farm/household resources available etc. Therefore, the objectives of this research were to describe milk production, marketing and processing practices of dairy cattle in Debremarkos Woreda and assess the current dairy cattle production and reproduction levels in the study area.

Materials and Methods

The study area

The study was conducted in Debre-Markos Woreda, which is located in Eastern Gojam Zone of Amhara National Regional State. Debre-Markos is found at 10020'N and 37040'E and the elevation is 2411 m above sea level. Debre-Markos is the capital city of Eastern Gojam Zone and is located at a distance of 300 km northwest of Addis Ababa and 265 km from Bahirdar, which is the capital city of the Region. The Woreda has seven (7) kebeles. The climatic condition of Debre-Markos is Woinadega, with mean annual temperature of 14.5°C and a range of 13.2°C in July and August and 17.3°C in March. Mean annual rainfall is 1300 mm. More than 75% of the total rain falls in the months of June, July, August and September locally known as 'kiremt' season. The driest months are November, December, January and February locally known as 'bega' season, when less than 5% of the annual total rainfall occurs. Debre-Markos's economy depends largely on agriculture. Considered to be a high potential crop- livestock zone and where dairy activity plays a significant role in the livelihood of the farming community. The human population of Debre-Markos is 107,684 out of these 101, 983 (54,928 female and 47,055 males) live in the towns while 5, 701 (2,863 female and 2,838 males) live in rural areas (Figure 1).

The study population

Households possessing dairy cattle in Debre-Markos Woreda and cattle owned by these households represented the study population.

The study design

A cross-sectional and retrospective type of studies were conducted using questionnaire survey, participatory methods and observation to collect data on characteristics and practices of smallholder dairy production systems from October 2007 to April 2008.

Sample size determination and sampling procedure

The sample size was calculated according to the formula recommended by Poet [8]: N=(ZC/X) 2 Where N is the estimated sample size; Z is the confidence level C is coefficient of variation in the population and X is the accuracy level. With 95% of confidence level, 50% coefficient of variation and 7% of accuracy level, a total of 196 households were required for the study. Thus, 200 households were selected randomly for this study, from which 132 households were selected from rural areas and 68 were selected from urban areas based on the proportion of households possessing dairy cattle available in each area.

Methods of data collection questionnaire survey

Detailed structured questionnaire was prepared and used to collect information through interviewing household head or in his/her absence, the most senior member available or the household member responsible for the farm. The questionnaire was pre-tested to check clarity and appropriateness of the questions and corrected when it was necessary. Some of the information collected through interview was supported by observation.

The data collected through interview was divided into sections covering: demographic characteristics, family size and composition, land holding and use pattern, livestock herd size and composition, objectives of dairy farming, division of labor in dairy production, housing condition of dairy cattle, dairy cattle feeds and feeding systems, breeding practices, record keeping, milk marketing and processing, reproductive and productive performance of dairy cattle, dairy cattle health problems and constraints.

Participatory appraisal

A total of five groups were formed, each containing 8-12

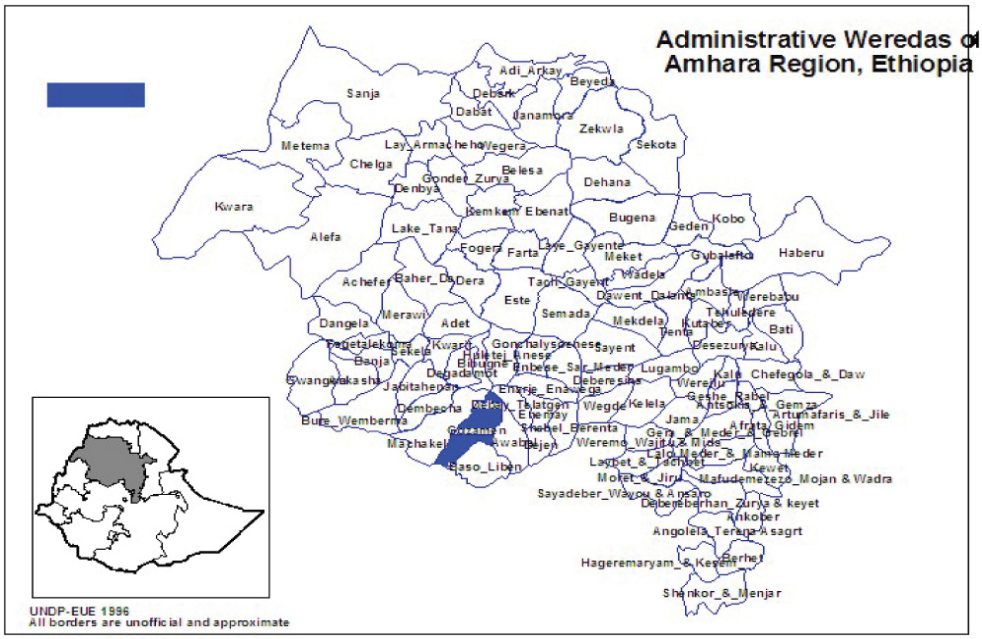

Figure 1: Map of the study area.

individuals. The group constituted both men and women members of the community, community leaders, livestock experts and Kebele development agents.

The information generated thorough the participatory method were constraints of dairy cattle production.

Personal observation

Data collected through observation of the farm environment were assessing housing conditions, feeding and feed storage practices, daily milk yield of dairy cows, livestock number and health status of animals and availability of farm records.

Data analysis

The data collected from the study was entered into Microsoft–Excel-spread sheet computer program and analyzed using SPSS statistical software computer programs (version 15, 2006). Descriptive statistics like percentage, mean, standard deviation and frequency distribution were used to describe the farming system characteristics in the study.

Results

Demographic characteristics

Table 1 shows the demographic characteristic of the sampled households in Debre-Markos Woreda. The overall average family size in Debre-Markos "Woreda" was 5.89 persons. The average family size was almost the same, 5.87 and 5.90 persons in urban and rural areas, respectively. From the overall sampled households, 83.5% were male headed households and the rest (16.5%) were female headed households. The highest percentage of female headed households was found in urban areas (23.53%) and the lowest percentage was found in rural areas (12.87%). Among the interviewed households, all urban area dwellers were engaged only in livestock production activity while in rural areas almost all (99.24%) were engaged in both crop and livestock production activities. Assessments of educational level of household heads indicated that generally, illiterate, junior secondary school, and high school and above constitute 38.0%, 43.5% and 18.5% of the respondents, respectively. In the urban area, the proportions for illiterate, junior secondary school, high school and above were 19.12%, 36.76% and 44.12%, respectively and in rural areas it was 47.73%, 46.97% and 5.30%, respectively. The majority (54%, n=108) of the household heads s were between 41-64 years of age. The higher proportion of households (54.41%) in urban area had a farming experience of less than 10 years as compared to those in rural areas (20.45%).

Land holding and land use pattern

The average land holding in the rural areas was 0.98 hectare with land size ranging from 0.25 to 4 hectares. The land use pattern in the rural area showed that nearly all of (96.96%) the legally tenured land was used for crop production and the remaining 1.54 and 1.50% of land was used for plantation and natural pasture, respectively. The plantation area is mainly covered by eucalyptus tree and Gesho. The major crops produced in the rural areas of the study site were oat (Avena sativa), «teff» (Eragrostis teff) and wheat which cover 30.29%, 29.26% and 20.22% of the croplands in the rural areas of the study site. The other crops grown in the study area were maize, barely, «noug» (Guizotia abbyssinica), linseed, beans and peas which cover 8.54, 5.53, 2.54, 1.03, 1.03 and 0.99% of cropland in the study area, respectively [11].

The majority of the households (92.65%) of urban area were land less. Only 7.35% of urban area dairy farms (n=5) have legally tenured land. The land holding of those households ranged from 1-10 hectares.

Livestock and cattle herd composition

The average livestock herd size and composition in the study area are indicated in Table 2. The average livestock holding per house hold in Debre-Markos Woreda was 7.32 TLU with an average livestock holding of 5.32 TLU and 8.36 TLU in urban and rural areas, respectively. The average cattle holding per house hold in Debre-Markos Woreda was

Variables and categories		Urban area (n=68) % (frequency)	Rural area (n=132) % (frequency)	Overall (N=200) % (frequency)
Sex of house hold head	Male	76.47(52)	87.12(115)	83.50(167)
	Female	23.53(16)	12.88(17)	16.50(33)
Type of agriculture	Livestock only	100%(68)	0.76(1)	34.50(69)
	Crop and livestock	-	99.24(131)	65.50(131)
Age of house hold head	Less than 40 years	11.76(8)	31.06(41)	24.50(49)
	41-64 years	58.82(40)	51.52(68)	54.00(108)
	>64 years	29.42(20)	17.42(23)	21.50(43)
Level of education	Illiterate	19.12(13)	47.73(63)	38.00(76)
	Primary and junior secondary school	36.76(25)	46.97(62)	43.50(87)
	High school and above	44.12(30)	5.30(7)	18.5(37)
Experience of dairy farming	<10 years	54.41(37)	21.22(28)	65(32.50)
	11-20 years	38.24(26)	9.39(52)	78(39.00)
	>21 years	7.35(5)	39.39(52)	57(28.50)

Table 1: Demographic characteristics of dairy farm owners in the study areas.

Livestock species	Urban area (n=68)		Rural area (n=132)		Overall (N=200)	
	Mean (SD)	%	Mean (SD)	%	Mean (SD)	%
Cattle	5.26	98.86	7.73	92.44	6.89	94.08
Sheep	0.03	0.55	0.24	2.87	0.16	2.33
Horse	0.02	0.44	0.09	1.09	0.07	0.94
Donkey	-	-	0.27	3.27	0.18	2.49
Poultry	0.01	0.15	0.03	0.33	0.02	0.16
Total	5.32	100	8.36	100	7.32	100
SD: Standard Deviation; TLU: Tropical Livestock Unit; 1TLU: 250 kg adopted from [1]						

Table 2: Average livestock holding (in TLU) per household in the study areas.

6.89 TLU with average cattle holding of 5.26 TLU and 7.73 TLU in urban and rural areas, respectively. In both rural and urban areas, the livestock herd was dominated by cattle. Next to cattle donkeys, sheep and poultry comprised only a small proportion of livestock herd in the study area; their proportion being higher in rural areas. Donkeys were found only in rural areas. The goat population in both urban and rural areas was almost zero and was not included in the calculation.

Cattle herd composition by breed

The cattle breeds found in Debre-Markos Woreda were local breeds (short horned zebu) and Holstein-Friesian × (short horned zebu) crossbreeds. The cattle herd size and composition in the urban and rural areas is indicated in Table 3. The mean cattle herd size per household was 7.35 and 11.60 in urban and rural areas, respectively. The range was from 2-37 cattle in urban areas and from 2-45 cattle in rural areas. The cattle herd composition in urban and rural areas was dominated by cows which was 41.20% and 29.00% in urban and rural areas, respectively. Crossbred cows comprised 31.1% and 3.38% in urban and rural areas, respectively. Average cow holding per household was 3.03 and 3.36 in urban and rural areas, respectively. The proportion of steer was higher next to cows in rural areas (20.31%) and extremely few in urban areas (0.6%), which were kept for fattening purpose. Average oxen (steer) holding per house hold was 2.36 in rural areas 0.02 in urban areas.

Division of labor in dairy production

Family labor was involved in 91.18% of urban dairy farms and in all rural dairy farms in dairy activities. Cattle herding was done by hired laborer in 48.53% of urban dairy farms and 29.55% of rural dairy farms for whom 2 birr per cow is paid monthly for herding cattle in communal areas in both rural and urban area farms. As reported by farmers during group discussion, individual interview, and field observation, the allocation of labor in the area is usually determined by the composition of the household. Livestock herding mostly was undertaken by the children and whose age was between 6 to 14 years of age. And adult males and females also herd cattle during the absence of children. Feed collection, milking, health monitoring, selling animals were done by both adult males and females. Other activities such milk processing, cleaning cattle shed, selling of milk and milk products, cow dung making and calf management were performed mostly by females.

Milk production, marketing and processing practices

Milking was two times per day during morning and evening except 4 farms in urban areas and 2 farms in rural areas, which practiced three times per day. Detailed information of milk marketing and processing is shown in Table 4 below. Results indicated that four types of milk and milk products were sold to consumers. These were milk, butter, fermented milk (yoghurt) and cheese. Butter was the most commonly sold milk product in the study area (93%) of the farms followed by raw milk (36%). In urban area 85.29%, 45.58%, 5.88%, and 4.41% of dairy farm owner practiced butter, raw milk, fermented milk and cheese, respectively. In the rural areas only butter and raw milk selling was practiced by 96.96% and 31% of the dairy farms, respectively.

Among households selling raw milk, 93% and 8% of the households sold directly to consumers and hotel (restaurants), respectively. The price of whole milk varied in urban and rural areas. In rural areas whole milk was sold at 3 birr per liter and in urban areas it was sold at 4.5 birr per liter. 1 kg of local cheese was sold at 8 birr per kg. About 92% of farm households practiced milk processing. Among milk processing households 99% use traditional milk processing equipment (clay pot). Most (92%) of farm the households processed raw milk at least to sour

Urban area (n=68)		Rural area (n=132)		
Variable	Mean	%	Mean	%
Herd size	7.35	100%	11.60	100%
Cows	3.03	41.20	3.36	29.00
Local	2.12	69.90	3.25	96.62
Cross	0.91	31.10	0.11	3.38
Heifers	1.41	19.20	2.22	19.20
Bulls	1.30	17.60	1.93	16.65
Calves	1.59	21.60	1.72	14.83
Steers	0.02	0.60	2.36	20..31

Table 3: Cattle herd size and composition in the study areas.

Milk products sold	Urban area % (frequency)	Rural area % (frequency)	Over all % (frequency)
Raw milk	45.58 (31)	31.00 (41)	36.00 (72)
Fermented milk(yoghurt)	5.88 (4)	-	2.00 (4)
Butter	85.29 (58)	96.96 (128)	93.00 (186)
Cheese	4.41 (3)	-	1.5 (3)
Raw milk sellers to consumers	83.87 (26)	97.56 (40)	93.05 (67)
Raw milk sellers to Hotels and Rest.	16.12 (5)	2.43 (1)	8.33 (6)
Milk processing house holds	82.35 (56)	96.96 (128)	92.00 (184)
Use modern processing equipment	3.57 (2)	-	1.08 (2)
Use traditional pot clay	96.24 (54)	100 (128)	98.91 (182)

Table 4: Types of milk and milk product marketed and milk processing practice in the study areas.

milk, butter, ghee, soft cheese and butter milk. And small proportion of households processed also local cheese into a product locally known as 'metata» which stays for a long time (one year) without spoilage specially during the long fasting period. Metata, ghee and buttermilk were used only for home consumption in the area [12].

Conclusion

The study was conducted to characterize dairy cattle production systems/practices/, to provide baseline data and identify constraints and opportunities for dairy cattle production in the study area The results from this study reveal that:

In both urban and rural areas of Debremarkos the livestock composition is highly dominated by cattle which show the greater contribution of cattle through better management, feeding, and genetic improvement and improvement of market infrastructures. Dairy cattle production systems in the study area can be grouped as rural crop livestock mixed farming system, land less intra urban dairy farms and urban specialized dairy arms. The legally tenured land holding in the rural areas was almost one hectare and from this 96.96% was used for crop production and only 1.5% was used for natural pasture which shows that crop residues are the major animal feeds in the study area. All rural people used communal grazing land as a source of animal feed which is the other source of animal feed The communal grazing land was utilized with differed grazing system for the wet season and free grazing system in the dry season but due to the swampy nature of the grazing lands and free grazing utilization during the driest periods tramping of forage and less productivity of the communal gazing land was identified as the major cause for feed shortage which shows the need for improvement of communal grazing land utilization. Urea treatment of crop residues and forage production are not practiced in the study area. In urban areas most of the farms used communal grazing land as a major source of animal feed in which animals were also supplemented with purchased crop residues, hay, concentrates and non-conventional feeds. This shows better feeding system of animals in urban areas as compared to rural areas.

Most of the farms 69% of rural area and 54.42% of urban area do not have raw milk selling practices due to lack of market access and small volume of milk produced and lack of milk market. Milk marketing system in the study area was contractual type of informal marketing system which lasts only to the non-fasting periods and milk marketing was stressed as a primary constraint by specialized dairy producers in urban areas who produce more milk (15-80 L) per day and also mentioned as a constraint by rural far distant farm households due to the long distant to transport milk on foot to urban areas for sell. This shows the need to improve milk market infrastructures for the improvement of dairy production in the study area. The majority of cows in the urban area 69.90% and almost all (96.62%) dairy cows in rural areas were local cows. The productivity of local cows was very low. The overall mean daily milk yields for local and cross bred cows' were 1.50 ± 0.68 L and 7.3 ± 4.65 L per day respectively. The overall mean lactation length, for local and cross bred cows was found to be 8.87 ± 1.55 month and 8.56 ± 1.75 months respectively. The overall mean age at first calving in the study area for local and cross breed cattle was 56.28 ± 5.29 and 35.44 ± 11.15 months respectively. The overall reported average calving interval for cross bred and local cows were 13.94 and 20.74 months respectively. The performance of cross breed cattle was better than local breed cattle in daily milk yield, age at first calving, and calving interval in both rural and urban areas. It was found that milk yield of cross bred cows was greater than that of local bred cows nearly by fivefold. This means that five local cows are one cross bred cow in terms of daily milk yield. The performance of local breed cattle in the urban areas was significantly higher than the daily milk yield of local cows in the rural areas which shows better management also can improve the daily milk yield of dairy cattle. Natural mating was the only method used for dairy cattle breeding. Avery small proportion of households used cross breed bull service. Farmers reported that though there was higher preference of natural mating with cross breed bulls there is limited cross breed bull in nearby. They also mentioned that the reason for lack of AI utilization were an availability at the time of heat; lack of attention for dairy production improvement; heat detection difficulty in local cows; suspicion of calving difficulty of local cows. In the study area there were no public veterinary services. Only 7.5% households used modern health treatment. But the rest 92.5% of the households used traditional method of health treatment and animal disease was mentioned as the first constraint for dairy cattle production in the area. Farmers reported that veterinary services from private sectors were not effective and costly.

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Iron Status of Infants and Toddlers Age 6 to 18 Months and Association with Type of Milk Consumed from DNSIYC Secondary Analysis

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Abstract

This study compares the intake of different types of milk, iron, haem iron, non-haem iron, and Vitamin C intake among infant and children age 6-18 months of age from Diet and Nutrition Survey of Infants and Young Children (DNSIYC). Approach SACN 2010 recommendation, the Reference Nutrient Intakes (RNI) of iron and Vitamin C for infant and children age 6-18 months was investigated in this study. Moreover, Ferritin and haemoglobin investigated as well to assess iron status, subjects who donated bloods were included in this study to investigate their iron status, 473 subjects for haemoglobin, and 443 for ferritin. Different factors investigated on 2567 subjects, include age groups, sex, ethnicity, socioeconomic level, maternal education level, and different types of milk consumed. This study found significant difference for ferritin and haemoglobin in different levels, across different types of milk consumed, haem-iron, Vitamin C, age groups and maternal education level. While there were no significant differences across sex, ethnicity and socioeconomic level in all age groups.

Keywords: Infants; Vitamin; Nutrition; Health; Gastroenterology

Introduction

This dissertation examines the association between the type of milk consumed and iron status of infants and toddlers ages 6-18 months living in the United Kingdom (UK). It is based on a secondary analysis of the Diet and Nutrition Survey of Infants and Young Children (DNSIYC) commissioned by the Department of Health and the Food Standards Agency [1,2]. The literature review establishes the importance of this relationship in application to recommendations about feeding cow's milk to infants and toddlers in this age group. Using current standards adopted by the World Health Organisation (WHO), it defines Iron Deficiency Anaemia (IDA) in children under the age of 5 years, as determined by indicators including haemoglobin and ferritin plasma concentrations. It then reviews current evidence and guidelines for breast-feeding and cow's milk intake for infants and toddlers with a focus on the relationship of type of milk intake to iron status. Although some discussion of infants under the age of 6 months is necessary to establish an understanding of the increased dietary needs for iron that occur after the age of 6 months, the focus of the review is on infants aged 6-18 months. This will set the context for the present study undertaken, which is expected to add to the current evidence base for future dietary recommendations in this area.

Breastfed infants of normal birth weight under the age of 6 months have a low prevalence of IDA and do not require iron supplementation; however iron supplements of 1-3 mg per day are thought to improve neurodevelopment for low birth weight infants less than 6 months [3]. For formula-fed infants under the age of six months, iron-fortified formula prevents IDA and may improve neurodevelopment [4]. For infants ages 6-12 months, follow-on formulas with iron fortification prevents IDA, but evidence regarding neurodevelopment is contradictory [4]. In infants ages 4-12 months, IDA is prevented by feeding iron- rich complementary foods and avoiding unmodified cow's milk; iron supplements prevents IDA in populations with high prevalence of IDA but does not benefits populations with low prevalence of IDA [4]. For toddlers 12-36 months of age, there is a dearth of studies, but there is moderate evidence that IDA may be prevented by feeding iron-rich complementary foods and restricting unmodified cow's-milk intake to less than 500 mL per day [4].

Consistent with these findings, the European Society for Pediatric Gastroenterology, Hepatology, and Nutrition Committee on Nutrition, which had previously failed to make recommendations for iron intake, now make the following recommendations regarding infants and toddlers above the age of 6 months [4]: "Follow-on formulas should be iron fortified; however, there is not enough evidence to determine the optimal iron concentration in follow-on formula.

From the age of 6 months, all infants and toddlers should receive iron-rich (complementary) foods, including meat products and/or iron-fortified foods.

Unmodified cow's milk should not be fed as the main milk drink to infants before the age of 12 months and intake should be limited to <500 mL daily in toddlers."

Woldu et al. [5] concur with these recommendations, concluding from their review that the best prevention against IDA is to replace unmodified cow's milk with iron supplemented formula; avoid feeding infants unmodified cow's milk until the age of one year; and provide breastfed infants with iron supplements or iron-fortified cereal.

Methodology

Sampling

In January 2010 the Cambridge shire 4 Research Ethics Committee approved the DNSIYC as a portfolio adopted study, as most of the clinics were NHS Trust sites [2,3] Stage 1 of the DNSIYC primarily involved the selection of subjects. A sample of infants and young children 4 months up to 18 months were drawn from across the

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UK Child Benefit (CB) Records, with the addition of a Healthy Start boost sample using a multi-stage random probability design to ensure the sample was stratified by "Government). A major limitation of the DNSIYC was that the number of subjects included in the study, exceeded the number of participants who donated blood; this affects the statistical power of the results Office Region, Index of Multiple Deprivation (IMD) scores and population density" [2].

Data preparation

The DNSIYC collected information on infants starting at age 4 months. Although this study interested in iron status from ages 6-18 months, they were also interested in the effects of complementary feeding which many parents follow earlier than 6 months, contrary to recommended practice [4]. However, the present dissertation loaded its dataset from UK data archive, and limits its analysis to the population of interest, which is the infants and toddlers age 6-18 months. Therefore, the worksheet for analysis was first limited by excluding any infants younger than 6 months, for a total of 2683 participants. The original main worksheet file contained 2683 participants and 1861 variables. When children under the age of 6 months were excluded, the number of subjects in the edited worksheet contained 2567 subjects. SPSS program was used to compare means of Ferritin and Haemoglobin in two age groups in these variables: Recode age group, sex, ethnic groups, socioeconomic group, education group, and drink of milk group, Vitamin C including supplements, total iron including supplements, haem iron including supplements and non-haem iron including supplements.

The present studies only looks at Haemoglobin (Hgb) and ferritin frequency statistics and compare means with all above recoded variables, to test the null hypothesis that the means of Ferritin and Haemoglobin for all age groups, sex, mother education, socioeconomic level and type of milk are equal.

The significance and relationships between hemoglobin, ferritin and Vitamin C tested accordingly in the following variables, SPSS software used for data analysis:

1. Age;
2. Gender;
3. Ethnic group;
4. Socioeconomic level;
5. Maternal education level;
6. Type of milk used (breast milk, infant's formula, follow-on formula or cow's milk).

Ferritin variable was divided into 2 groups According to WHO [6] Ferritin cut-off point:

- Normal ferritin above 12 µg/L.
- Low ferritin below 12 µg/L.

Haemoglobin variable was divided into 2 groups (non-anaemic) and (anaemic which include; mild/moderate/severe anaemia), according to the WHO [7] Haemoglobin cut-off point:

- Non anaemia (11 g/dL).
- Mild anaemia (10–10.0 g/dL).
- Moderate anaemia (7.0–9.9 g/dL).
- Severe anaemia (≤7.0 g/dL).

An independent samples t-test is the statistical method used to compare the means of a continuous dependent variable between two unrelated groups, and assess whether there is at least a 95% probability that the difference found does not arise by chance.

Independent sample T-test, to test the null hypothesis that the means of two groups, group of Ferritin and group of Haemoglobin are equal in different variables: Recode age groups, sex, Vitamin C including supplements, total iron including supplements, haem iron including supplements and non-haem iron including supplements, in the two age groups. While the independent samples t-test is used to compare two groups, the one-way Analysis of Variance Test (ANOVA) compares two groups and above.

One way anova, to test the null hypothesis that the means of Ferritin and Haemoglobin in all population in the groups are equal in the these variables: Group of education, group of socioeconomic level, group of ethnic and group of drink milk.

Results

The study included infants aged 6-18 months. Though the original sample consisted of 2,567 subjects, subjects with missing information relating to any aspect of the study were excluded from the sample. Therefore, number of subjects was 473 for haemoglobin-related tests, and 444 for ferritin-related tests (Figure 1).

Age

An independent sample T-test was conducted in order to compare Haemoglobin levels in infants aged 6-9 months and infants aged 10-18 months.

The test was unable to find a significant difference in the haemoglobin level for the two age groups p=0.297 (Figure 2). While there was a statistically significant difference in the Ferritin level for infants the two aged groups p=0.013 (Figure 3).

Sex

An independent sample T-test was conducted in order to compare Haemoglobin levels in males and females. The test was unable to find a significant difference in the scores for males and females p=0.509. For

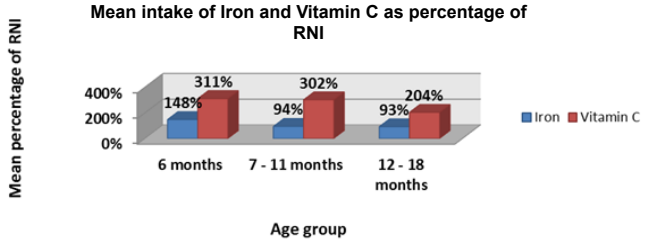

Figure 1: Percentage of vitamin C and iron means intake across the two age groups in comparison to Reference Nutrient Intakes (RNI), which include 2567 subjects.

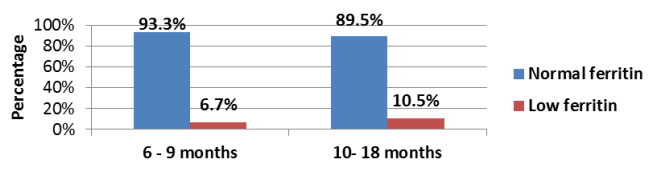

Figure 2: Ferritin percentage across age groups.

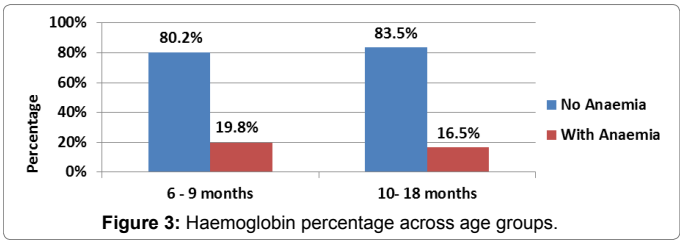

Figure 3: Haemoglobin percentage across age groups.

Ferritin, the test was unable to find a significant difference in the scores for males p=0.104.

Vitamin C

An independent samples T-test was conducted in order to compare Vitamin C levels in anaemic and non-anaemic subjects.

The test was unable to find a significant difference in the scores for anaemic subjects p=0.236, though the difference was close to being statistically significant. While for Ferritin, there was a statistically significant difference in the scores for low ferritin subjects and normal ferritin subjects p=0.022.

There was no statistically significant difference between the anaemic and non-anaemic subjects in levels of vitamin C, but there was a significant difference between subjects with low ferritin and normal ferritin; subjects with normal ferritin levels had significantly higher levels of vitamin C.

Dietary iron intake

An independent samples T-test was conducted in order to compare iron levels in anaemic and non-anaemic subjects.

The test was unable to find a significant difference in the scores for anaemic subjects and non-anaemic subjects P=0.796. There was no significant difference in the scores for low ferritin subjects and normal ferritin subjects p=0.310.

Dietary intake: Haem iron

An independent samples T-test was conducted in order to compare haem iron dietary intake in anaemic and non-anaemic subjects.

The test was find a significant difference in the scores for anaemic subjects and non-anaemic subjects p=0.014. A significant difference in the scores for low ferritin and normal ferritin subjects p=0.017.

Dietary intake: Non-haem Iron

An independent samples T-test was conducted in order to compare non-hem iron levels in anaemic and non-anaemic subjects.

The test was unable to find a significant difference in the scores for anaemic subjects and non-anaemic subjects p=0.861. There was no significant difference in the scores for low ferritin subjects and normal ferritin subjects p=0.26.

Ethnicity

A one-way analysis between subjects Analysis of Variance (ANOVA) was conducted in order to compare the effect of ethnicity on haemoglobin rates, comparing three groups: White, South Asian and other.

The test was unable to find a statistically significant effect of ethnicity on haemoglobin at the p<0.05 level; p=0.730. Moreover, ferritin at the p<0.05 level p=0.921. Ethnicity was not able to adequately explain the differences between subjects.

Maternal level of education

A one-way analysis between subjects ANOVA was conducted in order to compare the effect of parents' level of education on haemoglobin rates, comparing three groups: Degree level or above, qualification below degree level and no qualifications.

There was a statistically significant effect of parents' education on haemoglobin rates at the p<0.05 level; p=0.036. For ferritin, the test was unable to find a statistically significant effect of parents' educational level on ferritin at the p<0.05 level; p=0.574.

Socioeconomic group

A one-way analysis between subjects ANOVA was conducted in order to compare the effect of socioeconomic status on haemoglobin rates, comparing four groups: Managerial and professional occupations, intermediate occupations, routine and manual occupations, and not classifiable.

The test was unable to find a statistically significant effect of socioeconomic level on haemoglobin at the p<0.05 level, p=0.667. The test was unable to find a statistically significant effect of socioeconomic level on ferritin as well at the p<0.05 level p=0.932.

Milk consumption

A one-way between subjects ANOVA was conducted in order to compare the effect of consumption of different types of milk on haemoglobin rates, comparing five groups: Breast Milk, Infant Formula, and Follow on Milk, Cow's Milk or other.

There was a statistically significant effect of type of milk consumed on haemoglobin rates at the p<0.05 level; p=0.001. Moreover, there was a statistically significant effect of type of milk consumed on ferritin rates at the p<0.05 level; p=0.000 (Figures 4-7).

Discussion

Studies have shown that, during the first six months, infants who have been exclusively breastfed exhibited low iron intake, since human

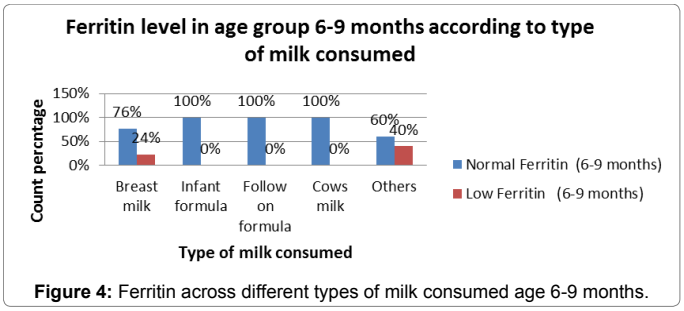

Figure 4: Ferritin across different types of milk consumed age 6-9 months.

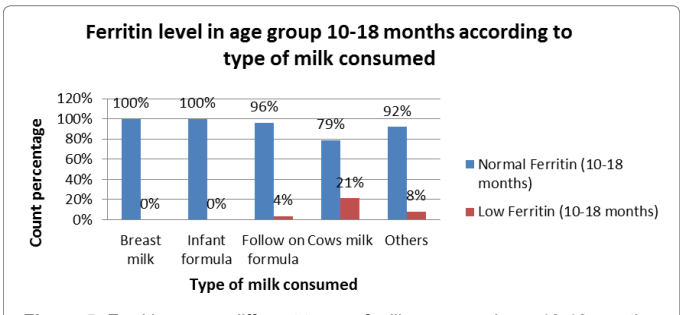

Figure 5: Ferritin across different types of milk consumed age 10-18 months.

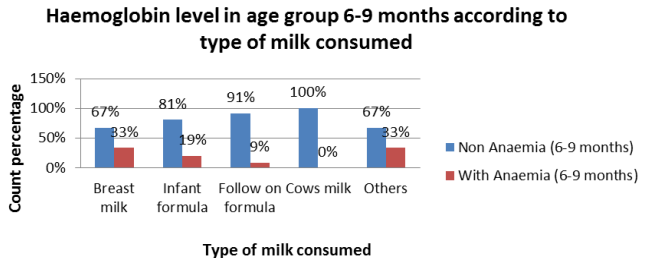

Figure 6: Haemoglobin across different types of milk consumed in age groups 6-9 months.

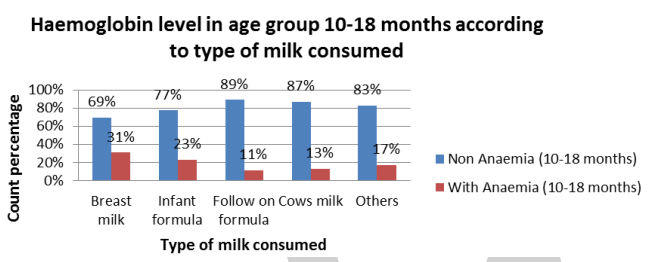

Figure 7: Haemoglobin across different types of milk consumed in age groups 10-18 months.

milk is relatively low in iron. At the age of 6 months, however, many infants are already receiving other forms of food, including specialised baby food and the beginning of solid food intake [7]. In addition, iron absorption undergoes developmental changes during infancy and early toddlerhood. Healthy adults have internal "regulators", such as dietary regulation, based on recent dietary iron intake, iron stores and other activity within the body. These regulators may not function at the same level or in the same way during very early infancy [8,9]. For instance, an iron-supplementation study of infants aged 4-9 months, in Honduras and Sweden, found that, at age 4 months, iron supplements were able to increase haemoglobin, regardless of the haemoglobin levels prior to the supplement intake. However, at the age of 6 months, the haemoglobin response was affected by the iron levels prior to supplement intake [10]. While these findings are compelling, the current study did not find a difference in haemoglobin levels, but rather it identified differences in ferritin levels, as younger infants had a higher level of ferritin. The current literature does not refer to this phenomenon.

In regards to gender differences, the current literature is inconclusive. Some studies have found that male infants may be more susceptible to anaemia, which is reflected in low haemoglobin levels, as well as lower rates of ferritin. For instance, one study conducted in an Asian population found that the risk for anaemia was 1.6 times higher for males than for females, and the risk for ferritin-deficiency anaemia was 3.3 times higher than for females [11]. However, other studies have failed to find any difference between male and female infants [12] the current study have approved the same finding, male and female have no difference in susceptible to anaemia.

Comparison of vitamin C and iron levels between the two levels of either haemoglobin or ferritin provides information regarding the possible association between levels of haemoglobin or ferritin and of vitamin C and iron. The study found an association between ferritin levels and vitamin C, in that higher ferritin levels were associated with higher vitamin C, but not with the haemoglobin.

Vitamin C plays an important role in facilitating the metabolism of iron, as well as the utilisation of iron for forming red blood cells. It has been posited that vitamin C may facilitate and stimulate the release of iron from ferritin-deposit storage [13] which may explain the positive association found in this study between vitamin C and ferritin.

There were no associations between iron, non-haem iron and ferritin or haemoglobin. Thus, haem-iron is the factor that is associated with ferritin and haemoglobin levels, and therefore this should be the focus of iron-enhancing interventions.

To this extent, studies have linked low serum ferritin concentrations to iron deficiency [14] and this may account for the association found in this research between iron levels and ferritin levels. The current study did not identify any significant association between total iron intake and haemoglobin or ferritin levels, though other studies have indicated that iron fortification of foods contributed to an increase in ferritin and haemoglobin alike [15], but this study has approved there is a significant association between haem iron intake and haemoglobin or ferritin levels. Therefore, future interventions intended to increase iron should focus on sources of haem iron rather than general sources of iron, seeing as non-haem iron has no effect on haemoglobin and ferritin.

This study shows that infant at age 7-11 months, mean intake of iron was 7.4 mg, 94% of recommended nutritional intakes RNI [16] 7.8 mg of iron, while infants at age of 6 months, mean consumption of iron was 6.4 mg, which exceeds RNI [16] 4.3 mg at 148%. Within this study, these research findings explains why infants who received either exclusive breast feeding or infant formula, at age 6 months met and exceed the iron RNI, while infants above the age of 6 months, which is the age of complementary feeding, failed to meet the iron RNI [16,17].

For Vitamin C, the mean intake for all age groups exceeds the RNI. Subjects, who consumed higher Vitamin C, have higher ferritin level than those who consumed less Vitamin C.

Significant differences between groups were found when grouping according to level of maternal education for haemoglobin, but not for ferritin, and no difference was found across ethnic groups. Upon further investigation, the study found that the difference between mothers with degree level education and those with below degree level education accounted for the statistically significant difference in haemoglobin rates, as infants whose mothers had a below degree level exhibited higher haemoglobin levels. These findings may indicate the involvement of a third factor that mediates this connection. Some of the possible explanations include the existence of a relationship between work demands and the feeding habits among mothers of infants, as well as an association between educational level and the child's care during the day. Studies have found that infants attending day care are less likely to be anaemic, which is attributed to their dietary intake and an increased likelihood that they consume iron-supplemented food [18-21].

When subjects were grouped according to the type of milk they consumed, statistically significant differences emerged in both haemoglobin and ferritin rates. Subjects consuming cow's milk were significantly lower levels of haemoglobin and ferritin levels (mean levels were 86 and 81, respectively) and those consuming milk other than the types listed exhibited the highest rates of haemoglobin and ferritin (mean levels were 110 and 105, respectively). Infant formula accounted for differences in the ferritin but not haemoglobin levels (for ferritin infant formula was the second highest, and for haemoglobin it was in the middle), and breast milk and follow on milk accounted for differences in the haemoglobin, but not ferritin levels.

Interestingly, the association between ferritin levels and type of milk consumed differed between the two age groups; among the younger group (aged 6-9 months) 24% of the infants consuming breast milk had low ferritin levels, while none of the older infants (aged 10-18 months) consuming breast milk exhibited low ferritin rates. Infants consuming infant formula in both age groups did not exhibit low ferritin, and among the infants consuming follow on milk, 4% of infants aged 10-18 months exhibited low ferritin while all infants aged 6-9 months exhibited normal ferritin rates. Among infants consuming cow's milk, there were no individuals in the younger age group with low ferritin levels, but among the older group 21% exhibited low ferritin. Finally, among infants consuming other types of milk, 40% of the infants in the younger age group who consumed other types of milk had low ferritin levels, but among the older group only 8% exhibited low ferritin.

Therefore, the main question guiding this research, with reference to the effect of the type of milk consumed on iron levels, was answered by isolating the types of milk that affected haemoglobin and ferritin in a unique manner. Researchers, health professionals and nutritionists alike agree that unmodified cow's milk should not be the primary milk drink for infants under the age of 12 months, and that general intake should be limited [22]. This coincides with the findings in this research according to which cow's milk accounted for differences in haemoglobin as well as ferritin.

Moreover, the general recommendation, regarding infants whose main source of nutrition is infant formula, is that this formula should be iron-fortified and contain at least 4-8 mg/L of iron [23,24]. This may explain the findings in the current research according to which infants receiving infant formula had different levels of ferritin, but not haemoglobin, compared to infants fed other forms of milk [25].

Iron content in breast milk has a higher bio-availability than iron-fortified formulas (12-49% absorption), as stated above, this level is low compared to the infants' needs [26]. During the first 6 months of an infant's life, the iron needs are mainly supplied by iron stored at birth, seeing as infants born full term and at adequate weight the endowed iron stores that can make up for the deficient breast milk for 6 months. After this time it is recommended to supplement the infant's nutrition with iron-rich or iron-fortified food [27].

Genetic factors may also play a part in iron deficiency, especially those related to red blood cells or the metabolism, such as sickle cell, and therefore should also be considered [28].

This dissertation examines the association between the type of milk consumed and iron status of infants and toddlers ages 6-18 months living in the United Kingdom (UK). It is based on a secondary analysis of the Diet and Nutrition Survey of Infants and Young Children (DNSIYC) commissioned by the Department of Health and the Food Standards Agency. The literature review establishes the importance of this relationship in application to recommendations about feeding cow's milk to infants and toddlers in this age group. Using current standards adopted by the World Health Organisation (WHO), it defines Iron Deficiency Anaemia (IDA) in children under the age of 5 years, as determined by indicators including haemoglobin and ferritin plasma concentrations. It then reviews current evidence and guidelines for breast-feeding and cow's milk intake for infants and toddlers with a focus on the relationship of type of milk intake to iron status. Although some discussion of infants under the age of 6 months is necessary to establish an understanding of the increased dietary needs for iron that occur after the age of 6 months, the focus of the review is on infants aged 6-18 months. This will set the context for the present study undertaken, which is expected to add to the current evidence base for future dietary recommendations in this area.

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Organic and Conventional Coffee (*Coffea arabica* L.): Differences in the Content of Minerals and Studies in Healthy and Induced Cancer Rats

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Abstract

Coffee is one of the most important agricultural products in international trade. The agricultural management system may influence the chemical composition of the beans in addition to altering the bioavailability of nutrients essential for humans. Therefore, the concentrations of Cu, Fe, Zn and the proximate composition of powder and coffee infusions from beans grown under organic or conventional agricultural systems were evaluated. In addition, the effect of these products on hepatic mineral content *in vivo* was investigated, in healthy and induced cancer rats. Our results showed that the levels of Cu, Fe and Zn were higher in conventional coffee powder than in organic powder. However, despite these differences, the organic coffee had higher extraction yield for all infuses, and its infusion at 20% (w/v) had higher level of Zn than conventional infusion. These results were associated with the agricultural system used and the extraction process employed during the preparation of infusions. The conventional coffee provides more adsorbent compounds, decreasing the availability of this mineral in the beverage. In terms of the mineral content *in vivo*, the ingestion of diets prepared with infusions or coffee powder did not influence the hepatic content of Cu, Fe and Zn.

Keywords: *Coffee Arabica* L.; Organic; Conventional; Micro minerals; Hepatic composition

Introduction

Coffee is one of the most important agricultural products in international trade. In 2009-2010, the amount of *Coffea arabica* exported throughout the world was approximately 62 million bags [1]. The area of production of conventionally grown coffee has increased [2], and this growth causes environmental impacts. The excessive application of chemical fertilizers and agricultural defensives (quantity and frequency), which usually exceeds the retention capacity of the soil, causes an imbalance in the ecosystem. To minimize these impacts, there is a consumption incentive for organically managed products, which have high prices compared to conventional products, despite the designation of quality and certification [3].

The International Federation of Organic Agricultural Movements (IFOAM) defines organic agriculture as "*all agricultural systems that promote environmental, socially and economically safe production of food and fibers*"[4]. However, the use of organic matter for the fertilization of coffee can interfere in the equilibrium of metals in the soil because organic acids act as ligands for many metals and the soil composition of these elements is reflected in the food [5,6]. Some studies have demonstrated differences between organic and conventionally produced coffee in terms of nutrients [7,8]. Among the nutrients, Cu, Zn and other toxic elements, such as Cd and Cr contained in some inorganic and organic fertilizers, can be increased in concentration in the soil and consequently be taken up by the plants [5].

Coffee consumption occurs mainly in the form of an infusion, resulting in one of the most appreciated and consumed beverages in the world [1]. The chemical composition of the drink is quite variable and is largely dependent on the species used [9-11]. and the system of management [2,5,7]. The concentrations of Fe, Cu and Zn, which are of acknowledged nutritional importance, in the infusion are the consequence of their levels in roasted beans, their physical-chemical characteristics (sorption) and the preparation conditions (filter paper and concentration).

Coffee is often consumed for its stimulatory effects owing to its phytochemistry, such as caffeine (the most prominent) [12], chlorogenic acid, lignans and some minerals components, which possess therapeutic potential, providing protection against cardiovascular diseases [13], diabetes mellitus [14], Parkinson's disease [15], Alzeheimer's disease [16], carcinogenesis [17] and antioxidants [7], as also observed in some drug treatments [18,19]. These aspects are highlighted since the coffee holds second position in consumption among all beverages after water [20].

However, some compounds in coffee like caffeine interact with some xenobiotics especially in women taking hormones to cure postmenopausal problems, in addition to other such as melanoidins and phenolic polymers, which can interact with the minerals present in the diet, interfering with their bioavailability [21,22].

Cu, Fe and Zn are essential micro minerals, whose absorption is relatively low and bioavailability subject to dietary, physiological and pathological factors, e.g. gastrointestinal tract cancers [23]. Besides, the neoplasms are also related to Zn and Fe deficiencies in animals and humans [24-26]. However, other studies have shown that higher

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levels these micro minerals in diet also can affects the tissues, such as liver [27,28]. Therefore, the aim of this work was to evaluate the concentrations of Cu, Fe, and Zn in coffee (powdered and infusions) from two different management systems (organic and conventional), and to investigate the hepatic mineral content in healthy and induced cancer rats, fed with these coffees.

Materials and Methods

Coffee samples and chemicals

To minimize the influence of environmental factors in the analysis, all samples of coffee were produced in the same geographic region and were harvested during the 2008-2009 season. So, any differences found can be attributed predominantly to the genotypic characteristics of the coffee and to the agricultural management style [7]. The samples of ground roasted coffee were from the 2008-2009 crop, Catuai red variety, and medium-roasted (200 - 215°C) for 15 min. The organic and conventionally grown samples were provided by the Association of Small Producers of Poco Fundo, Minas Gerais, Brazil, and the organic coffee was certified (BCS - OKO Garantie Master Certificates No POCO-7569/07.08/14291-BR). Fertilization of the organic coffee was with castor cake, and the conventional coffee was fertilized with nitrogen, phosphorus and potassium (20:5:20).

All solutions were prepared with analytical grade reagents and deionized water (Milli-Q, Millipore, Bedford, MA, USA). For the preparation of the samples and standards, the following chemicals were used: Zn (1000 mg L^{-1}), H_2O_2 30% (v/v), ferric chloride hexahydrate as the Fe stock solution (1000 mg L^{-1}) and cupric sulfate prepared as the Cu stock solution (1000 mg L^{-1}), which were obtained from Merck (San Diego, CA, USA); nitric acid (65% v/v) (Sigma-Aldrich, Steinheim, Germany). Laboratory glassware was kept overnight in a 10% (v/v) nitric acid solution. This glassware was rinsed with deionized water and freshly dried in a dust-free environment before use.

Proximate composition of coffee samples

A compositional analysis of all the samples (moisture, fat, protein, ash and carbohydrate) was performed in triplicate. Moisture was determined by loss while drying with an Infrared IV200 Moisture Analyzer (Gehaka) for 8 min at 120°C [29]. The total nitrogen content was analyzed by the Kjeldahl procedure (the conversion factor was 6.25 to protein), and carbohydrates were calculated to the remainder (the difference using the fresh weight-derived). The ash content was determined by the incineration of samples at 550°C in a muffle furnace. Fat content was measured in a Soxhlet system by extraction with solvent [30].

Sample preparation and Cu, Fe, Zn determination

The infusions were prepared in amounts equivalent to the average coffee consumption of the population (10% w/v), its half (5% w/v) and its double (20% w/v). Thus, 5, 10 and 20 g of coffee were added to 100 mL of water at 90°C and followed by vacuum filtration using qualitative paper filters (Framex, model 389/3) [7]. The infusions were reduced to1/4 of the total volume at 60°C.

The rats liver and experimental diets were homogenized and dried at 105°C, until reaching constant weight [30]. For the destruction of organic matter, the microwave-assisted acid digestion was used (Millestone® Ethos Plus microwave digestion system, Sorisole, Bergamo, Italy). Before digestion, all the samples were conditioned with HNO_3 and H_2O_2 for 30 min at room temperature. The heating program utilized (sample masses (n=4), the volumes of HNO_3 and H_2O_2) is described in Table 1. The samples digested were transferred to glass volumetric flasks, and the volume was brought to 25 mL with deionized water (Table 1).

The analytes Cu, Fe and Zn were determined using a flame atomic absorption spectrometer (FAAS) Shimadzu® AA-6800 (Chiyoda-ku, Tokyo, Japan) equipped with a deuterium background corrector. The hollow cathode lamps (Perkin-Elmer) for Cu (λ=324.8 nm), Fe (λ=248.3 nm) and Zn (λ=213.9 nm) were used. The operation conditions for FAAS were those described in the apparatus manual. The air-acetylene gas mixture was 2.0 - 1.8 mL min^{-1} for all analytes. For the standard preparations, the salts were dried at 105°C for 12 h and then cooled in a desiccator. For analytical curves, the standard concentrations for Cu and Zn were 0.1 - 2.5 mg L^{-1} and 0.5 - 6.0 mg L^{-1} for Fe. The limits of detection (LOD) and the limits of quantification (LOQ) were calculated according to the procedures of the *International Union Pure and Applied Chemistry* [31], and the accuracy was verified using a recovery test in all samples by adding an amount of standard equal to 1.0 mg L^{-1} [32].

Animal experiments and the isolation of liver samples

One hundred and forty animals were randomly allocated into

Samples	Quantities of samples	HNO_3 (mL)	H_2O_2 (mL)	Digestion program				References
				Steps	Time (min)	Power (W)	Temperature (°C)	
Coffee powder	300 (mg)	7	1	1	5	750	120	45
	(n=4)			2	3	750	120	
				3	10	750	210	
				4	15	750	210	
Coffee infusion*	2 (mL)	5	1	1	5	400	80	46
	(n=4)			2	5	400	120	
				3	5	400	210	
Rat livers and diets	300 (mg)	6	2	1	3	250	130	46
	(n=4)			2	5	630	130	
				3	22	500	130	
				4	15	0	130	

*All infusion volumes were reduced to 1/4 of the initial volume.

Table 1: Conditions of digestion program used in coffee samples, rat livers and diets.

Group/Treatment	nº of rats	chemicals	form of coffee applied	diet
G1/commercial	10	DMH	-	CD
G2/organic	10	DMH	5% infusion	ORC 5%*
G3/organic	10	DMH	10% infusion	ORC 10%
G4/organic	10	DMH	20% infusion	ORC 20%
G5/organic	10	DMH	powder	ORC 4%
G6/conventional	10	DMH	5% infusion	COC 5%
G7/conventional	10	DMH	10% infusion	COC 10%
G8/conventional	10	DMH	20% infusion	COC 20%
G9/conventional	10	DMH	powder	COC 4%
G10/commercial	10	EDTA	-	CD
G11/organic	10	EDTA	20% infusion	ORC 20%
G12/organic	10	EDTA	powder	ORC 4%
G13/conventional	10	EDTA	20% infusion	RCC 20%
G14/conventional	10	EDTA	powder	RCC 4%

DMH=1,2-dimetilhidrazine (40mg. Kg^{-1} body weight), a promoter of pre-neoplastic lesions in the colon. EDTA 1,5% (w/v) in NaCl 0,9% (w/v) (vehicle). CD=commercial diet; COC=diet with organic coffee; COC=diet with conventional coffee.*Considering the infusion embedded the feed (incorporated 100 mL infusion/kg commercial diet).

Table 2: Experimental design for the 12-weeks experiment to investigate the hepatic mineral content in healthy and induced cancer animals (DMH), fed with these coffees.

fourteen groups (*n*=10) (Table 2). The University Ethical Committee for Animal Research approved the protocols used in this study (protocol no. 235/09). We used 4-week-old male *Wistar* rats obtained from Centro Multidisciplinar para Investigacao Biologica (CEMIB) (UNICAMP Campinas, SP, Brazil). The animals were kept in polypropylene cages (5 rats/cage) covered with metallic grids and maintained at 22 ± 2°C, 55 ± 10% humidity and with 12 h light-dark cycles. They were fed commercial Nuvilab CR-1® diets (Nuvital Nutriente S/A, Colombo, PR, Brazil). For the preparation of modified diets, organic and conventional coffees in powder or infusion form were used. The infusions (5, 10 and 20% w/v) were prepared as described above. To incorporate the infusions in the commercial diet, a proportion of 100 mL of infusion per kg of commercial diet was used. This proportion allowed an appropriate pelleted diet. The significance of fiber as modulator of physiological and pathological processes prompted us to explore new potential applications. This way, the powdered coffee was incorporated in the proportion of 40 g powder per kg of diet [7]. After pelleting and drying, the conventional (COC) and the organic (ORC) coffee diets containing infusions of 5, 10 or 20% (w/v), or 4% (w/w) powder were obtained. These diets were fed to the animals for 12 weeks (Table 2).

To assay the effects of coffee in the animals with cancer, groups 1 to 9 received four injections of 1,2-dimethylhydrazine (DMH), a carcinogenic drug that induces pre-neoplastic colonic lesions. The doses administered were 40 mg kg^{-1} body weight, subcutaneous, twice a week, for two weeks (Table 2). In order to study animals without cancer, groups 10 to 14 received similar injections of the control vehicle [etilenediaminetetracetic acid (EDTA) 1.5% w/v] to simulate the stress of injection. At the end of this experimental step, all animals were anesthetized with ketamine and xylazine and euthanized by withdrawing blood from the heart. At necropsy, the livers were removed from all animals and stored at -18ºC until analysis [7] (Table 2).

Statistical analysis

In order to verify the differences between organic and conventional managements, the proximate (samples: coffee infusions, powders, diets) and micronutrient analysis (samples: coffee infusions, powders, diets and liver of rats), were compared using the Student's t test for paired data at 5% confidence level using the software *BioEstat*® 5.0.

The different mineral extractions from the infusions and the nutritional evolution of the animals (body-mass gain, diet consumption and consumption per animal per day of coffee powder) was examined with one-way analysis of variance (ANOVA/Tukey's test, $p<0.05$) using the software *Sisvar*® 5.1.

Results and Discussion

The results of the nutritional composition analysis revealed no significant differences between the powder and the infusions from either management system (Table 3). These results are in agreement with the Brazilian recommendation of National Agency of Sanitary Surveillance [33].

The micromineral contents were determined, and the LOD/LOQ for Cu, Fe and Zn were 0.065/0.215 mg L^{-1}, 0.127/0.424 mg L^{-1} and 0.024/0.081 mg L^{-1}, respectively. To check the accuracy, the recovery varied from 95 to 103% for all samples. The measurement precision, checked from variation coefficients of readings in triplicate, was always lower than 3% [32].

The conventional coffee powder contained higher concentrations of Cu, Fe and Zn than the organic powder (Table 4). This result can be attributed to the common use of chemical fertilizers in the conventional management system, which provide higher levels of N, P, K, S, Fe, Mn, Cu, B, Cd and Zn [5] compared to the natural fertilizer (castor cake) used in organic management systems [2,8]. These results demonstrate the influence of agricultural management in the amount and availability of minerals (Table 4).

Mineral composition of the powder and the coffee infusions (mg

Samples[a]	Carbohydrate	Ash	Fat	Protein	Moisture
ORC	58.30 ± 1.50	4.14 ± 0.27	18.76 ± 0.21	17.94 ± 0.56	0.81 ± 0.02
COC	58.36 ± 0.17	4.36 ± 0.01	18.59 ± 0.01	17.80 ± 0.07	0.79 ± 0.03
IORC 5%	0.10 ± 0.01	0.22 ± 0.01	0.10 ± 0.00	1.29 ± 0.33	98.54 ± 0.01
ICOC 5%	0.10 ± 0.01	0.21 ± 0.02	0.09 ± 0.01	1.37 ± 0.08	98.49 ± 0.21
IORC 10%	0.10 ± 0.02	0.41 ± 0.02	0.17 ± 0.02	1.58 ± 0.16	97.40 ± 0.02
ICOC 10%	0.10 ± 0.02	0.41 ± 0.01	0.19 ± 0.01	1.68 ± 0.09	97.42 ± 0.01
IORC 20%	0.11 ± 0.02	0.71 ± 0.06	0.31 ± 0.01	2.89 ± 0.17	94.96 ± 0.04
ICOC 20%	0.11 ± 0.02	0.73 ± 0.02	0.29 ± 0.01	2.68 ± 0.14	94.94 ± 0.01

COC=conventional coffee; ORC=organic coffee; ICOC=infusion conventional coffee; IORC=infusion organic coffee. No statistic difference by Student's t-test ($p<0.05$).

Table 3: Proximate analyses of powder and infusion (organic and conventional coffee) samples (mean ± SD) (g 100 g^{-1}) (*n=4*).

Samples	Cu	Fe	Zn
ORC	21.29 ± 0.47[a]	40.93± 0.88[a]	22.22 ± 0.59[a]
COC	25.03 ± 0.22[b]	45.70± 0.51[b]	27.08 ± 0.99[b]
IORC 5%	<LOD	<LOD	2.53 ± 0.12[a]
ICOC 5%	<LOD	<LOD	2.73 ± 0.12[a]
IORC 10%	<LOD	<LOD	2.50 ± 0.20[a]
ICOC 10%	<LOD	<LOD	2.27 ± 0.06[a]
IORC 20%	<LOD	<LOD	2.93 ± 0.03[a]
ICOC 20%	<LOD	<LOD	1.80 ± 0.03[b]

conventional coffee; ORC=organic coffee; ICOC=infusion conventional coffee; IORC=infusion organic coffee; LOD=limit of detection. [a,b] Statistical difference of powders and between each infusions concentration by Student's t-test ($p<0.05$).

Table 4: Mineral composition of the powder and the coffee infusions (mg kg^{-1}) (mean ± SD) (*n=4*).

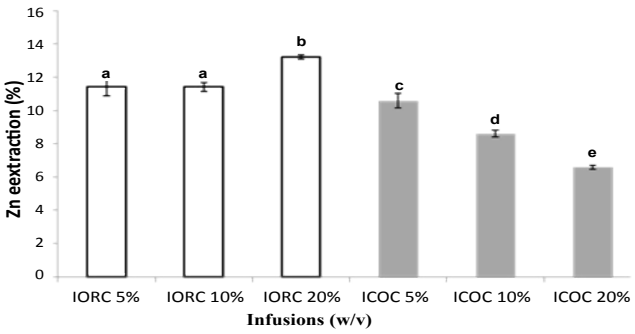

Figure 1: Zn extraction yield in organic and conventional infusions.

ICOC = infusion conventional coffee; IORC = infusion organic coffee; [a,b,c,d,e] Statistical difference among columns. [a,b,c,d,e] Statistical difference between the columns by ANOVA/Tukey's test ($p<0.05$).

kg^{-1}) (mean ± SD) (n=4).

In the infusion studies, we observed that although Cu and Fe were present in higher concentrations than Zn in the powdered coffee, they were poorly extracted by the infusion method and their concentrations were lower than the LOD, independently from the agricultural management system (Table 4). Therefore, they were strongly adsorbed in the powder and/or the paper filter. On the other hand, Zn was detected in all infusions. Furthermore, in the 20% (w/v) infusion prepared from the organic powder, Zn was present at higher levels than in the conventionally managed sample (Table 4).

We observed that although the conventional coffee powder presented higher levels of zinc than organic coffee powder, its extraction yield for all infuses was lower than organic coffee, as showed clearly in Figure 1. This fact can be explained by the relationship between the quantity of water and the powder mass used for each infusion, suggesting that coffee can act as an adsorbent. This may have occurred due to the inherent characteristics of the powder, which contains potential desorption/adsorption properties, mainly due to the roasting process, when the temperature of pyrolysis reaches 220°C. Therefore, the Zn level may be the result of the interaction of Zn with degraded compounds during the roasting of the coffee, *i.e.*, with the formation of insoluble ZnO in an aqueous solution [34] or by the interaction of Zn with hydrosoluble compounds of samples that may have eluted during filtration [35,36] This adsorbent property of coffee was used by Boonamnuayvitaya et al.,[37] who used the residues produced by the instantaneous coffee manufacturing to remove heavy metal ions in the solution (Figure 1).

The different mineral extraction efficiencies from the infusions may also be compared to the affinity of these elements in carbon-based adsorbents, which vary in the order of $Zn^{+2} < Fe^{+3}/Fe^{+2} < Cu^{+2}$, [38] and to their adsorption in filter paper, which varies in the increasing order of Zn (0.06%), Cu (2.11%) and Fe (5.34%) [39] According to Kononova et al.,[38] the ion affinities may be attributed to the different values of the stability constants of the compounds formed with the functional groups on the surface of the adsorbents. These affinities explain the higher extraction of Zn in the infusion compared to Cu and Fe.

In addition to the adsorption/elution properties and the extraction sequence, which are common to the infusions from both management systems, it is also concluded that coffee from the conventional management system provides more adsorbent compounds and decreases the availability of Zn in the infusion.

Comparing the Zn levels to the Recommended Dietary Allowance (RDA) [40], the usual consumption of 300 mL of a coffee infusion can supply the human body with up to 1.61% of the RDA, then the possible food replacement by coffee in improper diets cannot be performed.

Although the influence of elements of coffee complex matrix on interactions with macronutrients from diet such as fibers or protein, in the mouth or gastrointestinal tract, have been clearly demonstrated in many reports [41,42] a better understanding of these interferences in the diet is essential. For this purpose, we evaluated the possible effects of coffee matrix in the liver of healthy rats or cancer induced (treated with DMH) through the concentration of Fe, Cu and Zn. The infuses and ground coffee were incorporated in the diet for mimicking the common ingestion of coffee together other foods and their possible interactions.

The drug DMH used to induce colon cancer is metabolized in the liver to produce methyl free-radicals, generating hydroxyl radicals or hydrogen peroxide that can induce oxidative damage in vulnerable targets, such as DNA bases, resulting in cancer, inflammation and aging [43]. In this *in vivo* study, there were no significant differences in the composition of the diets with and without coffee; and the average concentrations in diets were as follows (g 100 g^{-1}): carbohydrate 45.5 ± 5.5, ash 8.8 ± 2.3, lipid 4.9 ± 0.4, protein 27.8 ± 6.4 and humidity 11.8 ± 3.5; and for minerals were (mg kg^{-1}): Cu 35.02 ± 0.72, Fe 113.72 ± 14.53 and Zn 0.16 ± 0.21. The compound levels in the experimental diets are consistent with those suggested by Ammerman et al. [44] and Oliveira et al. [45] and fulfill the needs of the animals. Besides, to ensure study homogeneity, the nutritional parameters of animals showed that there were no significant daily changes in the consumption of the diets (25.5 ± 3.1 g per rat) or in body-mass gain (26.12 ± 9.23 g per rat), for all groups. Based on the diet consumption, the animals from each respective group ingested coffee daily in the following amounts: 0.14 ± 0.01 g (infusion groups 5% w/v), 0.26 ± 0.03 g (infusion groups 10% w/v), 0.54 ± 0.04 g (infusion groups 20% w/v) and 1.03 ± 0.05 g (powdered coffee groups 4% w/v). The proportional consumption of coffee evidenced that the use the diet as vehicle is adequate for this kind of sample. Furthermore, the results showed that the all diets containing coffee (infuses or ground) did not influence the contents of minerals in the livers of all experimental groups, with the following mean values: 0.68 ± 0.05 mg g^{-1} for Fe and 0.14 ± 0.02 mg g^{-1} for Zn. Cu was not detected. As the changes in mineral concentrations in the liver, heart, plasma, bone and bile, are indicative of their bioavailability [46-48], we believe that the hepatic content of Cu, Fe and Zn did not suffer from the action of chelating substances, such as brown-melanoidin-like polymers derived from chlorogenic acids, sugars, proteins [22], and fibers in the coffee powder (4% w/w) or the neoplastic action of DMH.

Conclusion

Overall, our results support the conclusion that the mineral content of coffee is influenced by the conditions of agricultural management styles and the extraction process. The levels of Cu, Fe and Zn were higher in conventional coffee powder than in the organic powder. However, despite differences in the powder, the organic coffee had higher extraction yield for all infuses, and its infusion at 20% (w/v) had higher level of Zn than other conventional infusion. Regarding the mineral content *in vivo*, the ingestion of diets containing infusions or coffee powder did not influence the hepatic content of Cu, Fe and Zn.

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Calcium Delays the Postharvest Ripening and Related Membrane-Lipid Changes of Tomato

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Abstract

Effects of foliar $CaCl_2$ treatment on postharvest tomato (*Lycopersicon esculentum* Mill.) ripening and senescence as well as on membrane lipid degradation were assessed during two separately carried out experiments. In the first one, foliar $CaCl_2$ application on two cultivars Trésor and H 63-5, caused a significant increase in fruit Ca content and consequently slows the diminution of firmness and acidic citric content, the increase of pH, and the development of red color and gray mold during storage. In addition, $CaCl_2$ application had more effect on the softer H 63-5 fruit, which contained relatively low level of Ca at the time of treatment. In the second experiment calcium implication in cellular membrane stabilization of cultivar Caruso has been verified. Chlorophylls content decreased and that of the carotenoids increased during ripening and senescence, in correlation with of phospholipids reduction. In conclusion, the Ca delays tomato ripening and senescence during storage. This could be probably by protection of membrane lipids from degradation.

Keywords: Tomato fruits; $CaCl_2$; Ripening; Senescence; Storage; Lipid degradation

Introduction

The involvement of Ca in the regulation of fruit maturation and ripening is well established [1,2]. It has been reported that fruit containing low Ca level are sensitive to many physiological and pathological disorders, and consequently have short shelf-life [3-6]. Thus, Ca applying before/after harvest prevents physiological disorders, increase resistance to diseases, delays ripening and subsequently, improves quality of fruit crops [6-9]. Ca treatment has been shown to decrease respiration, reduce ethylene production and to delay the onset of ripening of apple, strawberry, avocado and mango [1,6,10-13]. Preharvest calcium sprays may slightly increase fruit calcium content and this increase may differ from year to year, depending on actual environmental factors [8]. Nevertheless, the success of attempts to increase Ca levels in some fruit by preharvest $CaCl_2$ spray has been limited [12,14].

Studies highlighting the mechanism of action of Ca showed that the Ca may affect the structure and function of cell walls and membranes and certain aspects of the cell catabolism [15-18]. Indeed, the loss of cell membrane integrity is characteristic of plant senescence [19]. This is evident from progressive ultrastructural deterioration and from increased leakage of solutes. Reduced membrane Phospholipids (PL) content during senescence is an index of membrane breakdown as shown for senescing cabbage leaves [17]. This effect can be delayed by Ca, either by preharvest or postharvest application [4]. However, some studies report acceleration of senescence [1,17]. The excessive increase in cytosolic Ca level could be stimulate lipolytic enzyme and accelerate deterioration of membranes [17,19]. Although, Ca effects on fruit senescence has been demonstrated, only few studies show the effectiveness of pre-harvest $CaCl_2$ treatment on the preservation of quality and the prevention of physiological disorders during the storage of tomato (*Lycopersicon esculentum* Mill.) at low temperatures. Indeed, after harvest, the fruit ripens quickly. This may be responsible for fruit short life and represents a serious constraint for efficient handling and transportation. Therefore, losses are often significant [20].

The present study compare the effects of foliar application of CaCl2 on fruit Ca content, fruit ripening and susceptibility to mold development of two tomato cultivars differing in firmness. In addition, this study investigates whether the delay of tomato ripening, caused by Ca treatment, was related to lipid membrane protection from degradation.

Materials and Methods

Two experiments were carried out to evaluate the $CaCl_2$ effects on tomato (*Lycopersicon esculentum* Mill.) preservation and cell membrane degradation. In the first experiment, where the Ca effects on tomato, cvs. Trésor and H 63-5, maturation and senescence was verified, plants were treated with $CaCl_2$ by foliar application, 16, 8 and 3 days before harvest at dose 0 or 10 kg/ha. They were fertilised according to the recommendations of the Ministry for the Agriculture of Tunisia. Plants were grown on a sandy-loam soil at a spacing of 60 cm between plants, 1 m between row, and 2 m between plots. Immediately after harvest, fruit was pre-cooled and selected for uniformity of size and color (one-forth to one-half red) and lack of wounding. Tomatoes were stored in 26-liter polyethylene containers under a continuous air flow at 12°C and close to 100% RH during 21 days in dark. They were 9 lots of 16 tomatoes in each container. The composition of the container atmosphere was checked by gas chromatography (model 29, Fisher-Hamilton Gas practitioner; Ottawa, Ont.). Ca was determined in fruit by atomic spectrophotometry (Perkin Elner, Analyst 300, USA). The fruit samples were dried at 70°C and digested with nitric and perchloric acids [21]. Ripening after harvest was assessed by measurement of free sugar, titratable acidity, pH, color, firmness and by visual rating of

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mold development. For determination of free sugar, titratable acidity and pH, eight fruit homogenates per replication were filtered through layers of cheese cloth to obtain clear juice. Free sugar content was recorded by a refractometer (Bausch and Lomb optical series YB 3301; Bausch and Lomb, Rochester, N.Y.). Results were expressed as percent free sugars. Titratable acidity was determined as described by Morris et al. [22] by titrating the fruit juice, after diluting with distilled water, against 0.1N NaOH solution using phenolphthalein as an indicator to the end point at pH 8.1. Results were expressed in terms of percentage citric acid [23]. The pH was evaluated by pH-meter (Metrohom 744) according to the method described by Chéour et al. [24]. Electrodes were directly immersed in a juice. Fruit firmness was determined on 8 tomatoes per replicate, as described by Lana et al. [25] with Universal Testing Machine (Instron, model 4411, Canton, Mass., USA). Firmness was measured via compression using 0.05 kN load cell and stainless steel, 7.5 mm diameter convex probe. After establishing zero-force contact between the probe and the horizontally positioned fruit, specimens were compressed 2.5 mm at the equatorial region of each fruit. The maximum force (N) generated during the probe travel used for data analysis. Measurements were made at two equidistant points on the equatorial axis of each of 12 fruits. Color was determined on 8 fruits as tomato color index (TCI) according to the formulate a/L x [L/(a2 + b2)], of Hunter L, a, b method (Colorgard 1000; Pacific Scientific, Silver Spring, Md.). The hunter "*a*" value ranges from green (negative) to red (positive). The hunter "*b*" value ranges from blue (negative) to yellow (positive) [26]. Mold was estimated visually, using a scale from 0 to 9. 9 indicating completely mold covered fruits. Results are means of 16 tomatoes per replicate. All measurements were performed at 20°C.

In the second experiment, where the implication of Ca in the stabilization of the cellular membranes was verified, Phospholipids (PL), Free Sterols (FS) and Free Fatty-Acids (FFA) were determined during ripening of the cultivar Caruso. Plants were grown under the same conditions of the experiment 1. The layout of the plots was also similar. Plants were treated with 0 or 10 kg $CaCl_2$/ha at 9, 6 and 3 days before harvest. The storage has been made according to conditions described in the first experiment. The ripening of the tomatoes was evaluated by determination of chlorophylls (Chl) and carotenoids contents as described by Bergevin et al. [27]. Extraction was done by a chloroform and methanol mixture (2/1, v/v) and the absorbance was measured at 480 and 664 nm by a spectrophotometer (Halwett Packard, Model 8451A Diory Arry). Pure Chl B and lycopene solutions were used to plot the standard curve. Pericarps were fixed in boiling water for 3 min to inactivate endogenous phospholipases.

Total lipids were extracted from tissue conforming to Blight and Dayer procedure [28]. The lipids in the chloroform phase were separated by TLC on 250 µm silica gel G plates (Fisher Scientific Co., Ottawa, ON). Acetone/acetic acid/water (100/2/1, v/v) was used to separate the PL from galactolipids, hexana/diethyl ether/acetic acid (80/20/1; v/v/v) was used to separate the neutral lipids, and chloroform:methanol/acetic acid/water (80/15/15/3.5, v/v/v/v) was used to separate PL. The lipids were visualized in iodine vapors and identified using authentic standards (Sigma, St-Louis, MO). The area corresponding to each class on the TLC plate was scraped into a test tube and transmethylated directly onto the silica gel with 14% (w/v) BF3 in methanol [29]. For quantitative determination of FA, a known amount of heptadecanoate (C17:0) was added as an internal standard. Methyl esters of FA were analyzed by GLC (Hewlett-Packard, model 5890A, Mississauga, ON) on a 30-m capillary DB 225 column (J & W Scientific, Rancho Cordova, CA) as described by Makhlouf et al. [30]. FS were silylated directly on the silica gel [31] and assayed by GLC using cholestane as a standard. Sterol trisilyl derivatives were separated by GLC on a 25-m capillary column (Hewlett-Packard, ULTRA 1, Mississauga, ON). Lipoxygenase (LOX) activity was determined spectrophotometrically at 234 nm [32]. The standard assay mixture contained 1.5 mM linoleic acid and 0.5% (v/v) Tween 20 in 30 mL Pipes buffer (pH 7). A 0.5-mL aliquot of the extract was added to 2 mL of reagent in a cuvette.

Analysis Of Variance (ANOVA) of results was made following a factorial randomly complete block design [33] by the GLM procedure of the SAS statistical package [34]. The sources of variation were cultivar, $CaCl_2$ rate and time of storage, and their interactions for the first experiment. Homogeneity of variance was verified by the standard Bartlett test [35]. Each treatment was randomised on three blocks. The two experiments were repeated twice and only the results of the seconds are presented.

Results

Effect of Ca on tomatoes maturation

Foliar application of $CaCl_2$ caused an increase in Ca level of fruits in both cultivars (Table 1). However, the Ca content increased more in H 63-5, which had shown before $CaCl_2$ application a relatively lower Ca level, than in Trésor ($P<0.05$). Free sugar increased during storage for both tomato cultivars. This increase was not delayed by Ca for both cultivars ($P > 0.05$) (results not shown). Organic acids, expressed as citric acid, increased at the beginning of storage and decreased at the end in both cultivars (Figure 1). Ca treatment delayed this change but more so with H 63-5 ($P<0.05$). The pH increased for two cultivars during storage (Figure 2). The increase was delayed by $CaCl_2$ treatment for both cultivars but the effect was more marked for H 63-5 ($P<0.05$). The pH of tomatoes treated with $CaCl_2$ was more acid at harvest and during storage. Color, expressed in TCI, increased gradually for both cultivars to about the same level during storage (Figure 3). The effect of Ca was observed as from the 7th day of storage for both cultivars but it was more pronounced in the case of H 63-5 ($P<0.01$). With time of storage less force was required to compress the fruit (Figure 4). The decrease in firmness was delayed by calcium treatment for both cultivars. The effect of calcium treatment was more observed at harvest and throughout the storage period for H 63-5 ($P<0.05$). Mold developed more quickly on H 63-5 than on Trésor (Figure 5). Ca treatment caused a delay in mold development but the effect was clearer on H 63-5 ($P<0.01$). For all these maturity characteristics, the 'cultivar x Ca x storage' interaction was not significant which indicated that the cultivars responded in the same way to $CaCl_2$ treatment even if H 63-5 reaction were slightly more important ($P > 0.05$).

Effects of Ca on the membrane lipids degradation

Chl content of pericarp decreased significantly during storage of fruits at 12°C (Table 2). This decrease was accompanied by a significant increase in carotenoids content. However, $CaCl_2$ treatment delayed these changes. PL and FS contents were measured during storage to

Cultivars	$CaCl_2$ (kg/ha)	Ca content (mg/100g FW)
Trésor	0	10.4 ± 0.3
	10	11.0 ± 0.8
H 63-5	0	08.9 ± 0.6
	10	11.1 ± 0.5

$CaCl_2$: Dichloride Calcium; FW: Fresh Weight.

Table 1: Calcium content (%FW) of tomato pericarps, cvs Trésor and H 63-5, after foliar application of $CaCl_2$. Values are means ± SD for 3 replicates.

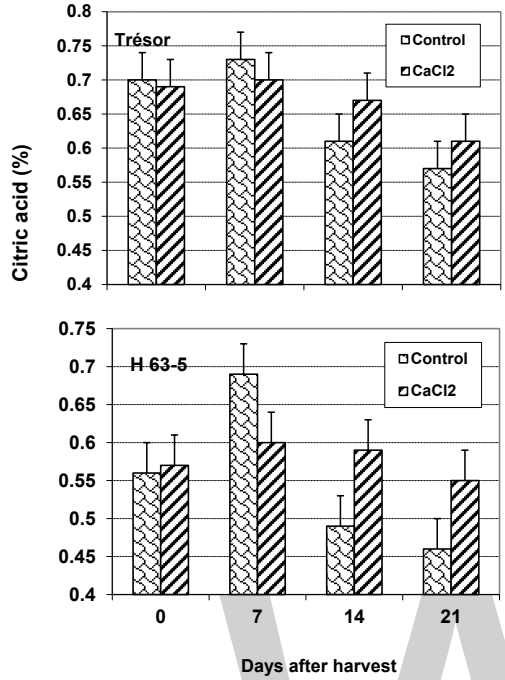

Figure 1: Titratable acidity (citric acid, %FW) of 'Trésor' and 'H 65-3' tomatoes stored in darkness under a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. Vertical lines show average SD for 3 replicates.

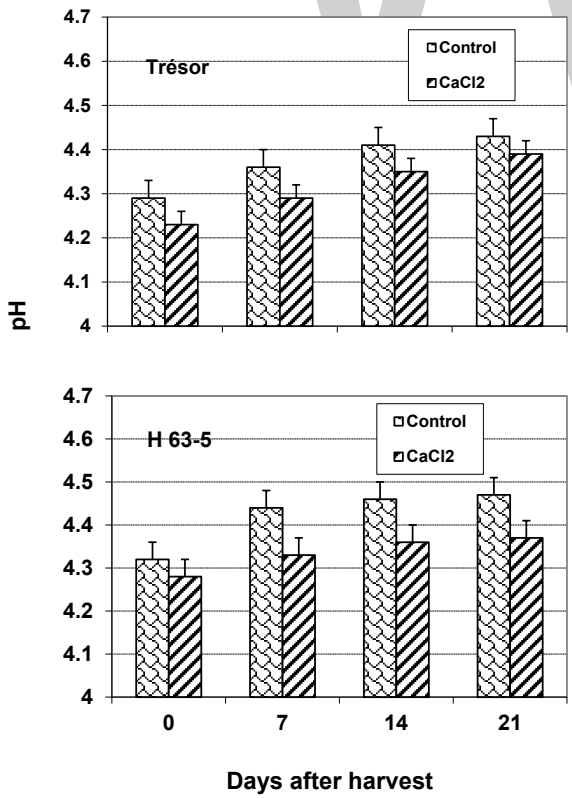

Figure 2: pH of 'Trésor' and 'H 65-3' tomatoes stored in darkness under a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. Vertical lines show average SD for 3 replicates.

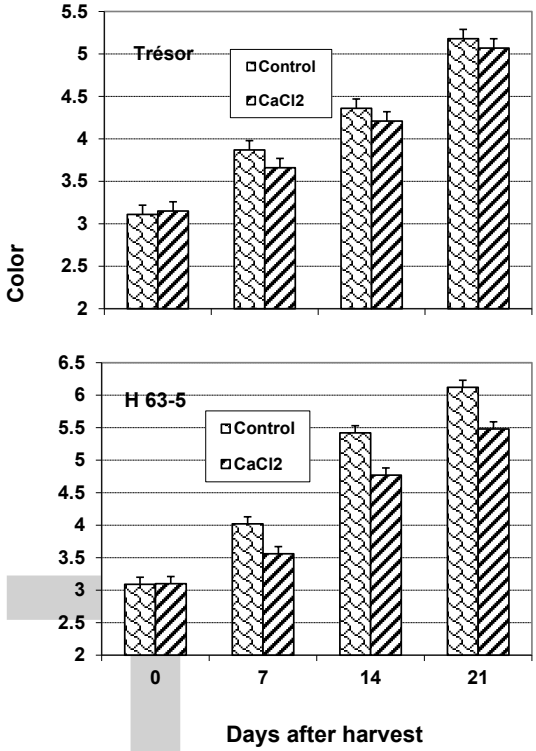

Figure 3: Color of 'Trésor' and 'H 65-3' tomatoes stored in darkness under a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. Vertical lines show average SD for 3 replicates.

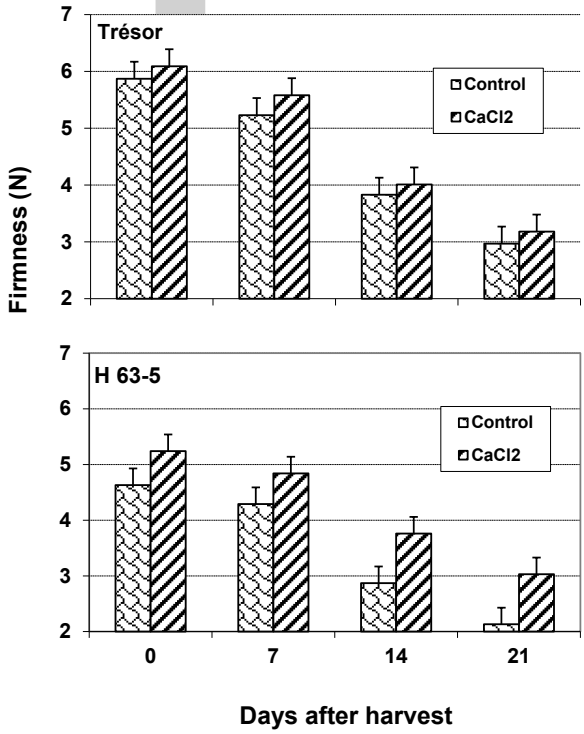

Figure 4: Firmness of 'Trésor' and 'H 65-3' tomatoes stored in darkness under a continuous air stream at 12°C and close to 100% RH for 21 days after foliar CaCl2 application. Vertical lines show average SD for 3 replicates.

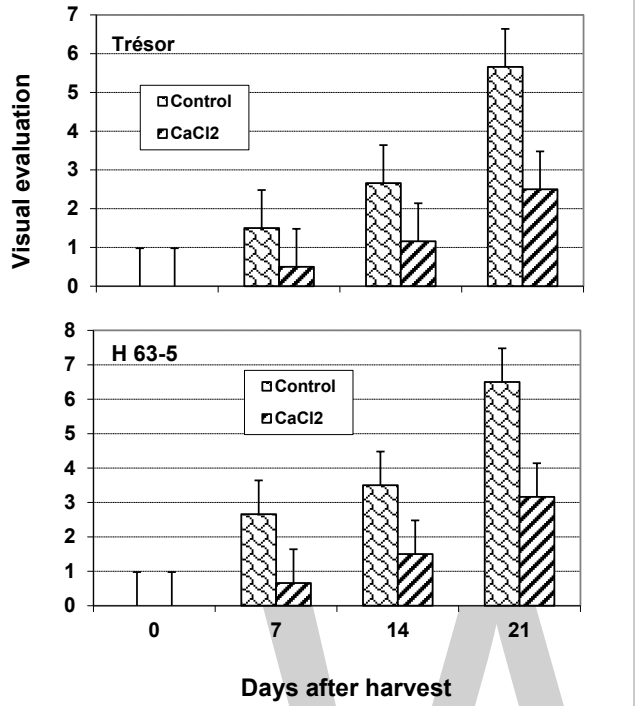

Figure 5: Visual assessment of mold development on 'Trésor' and 'H 65-3' tomatoes stored in darkness under a continuous air stream at 12°C and close to 100% RH for 21 days after foliar CaCl2 application. Vertical lines show average SD for 3 replicates.

Treatment	Days	Chlorophylls (nm/g FW)	Carotenoids (nm/g FW)	PL (nm/g FW)	FS/PL (µg / µg)
Control	0	10.6 ± 0.7	7.2 ± 0.09	179.2 ± 26.0	0.15 ± 0.01
	14	0.3 ± 0.1	92.0 ± 21.6	156.1 ± 33.2	0.26 ± 0.04
	21	≤ 0.1	167.2 ± 37.9	141.6 ± 36.6	0.37 ± 0.03
$CaCl_2$	21	≤ 0.1	167.2 ± 37.9	141.6 ± 36.6	0.37 ± 0.03
	21	0.9 ± 0.2	102.7 ± 21.3	166.1 ± 33.9	0.21 ± 0.02

$CaCl_2$: Dichloride Calcium; FS/PL: Free Sterol/Phospholipids; FW: Fresh Weight; RH: Relative Humidity; PL: Phospholipids.

Table 2: Change with time in Chlorophylls, Carotenoids and PL contents, and FS/PL ratio of pericarp tissue during storage in darkness of tomato fruit, cv. Caruso, in a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. Values are expressed as means ± SD.

determine whether the changes in Chl and carotenoids contents were associated with an alteration in membrane lipid composition. Total PL content declined during storage for all treatments (Table 2). The rate of decline in PL level was less important with fruit treated with Ca. Water fruit content does not change during storage. The most important PLs were phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositole and the phosphatidylglycerole (48, 37, 8 and 3%, respectively). The proportion of PL classes did not change significantly during storage which indicated that each class of PL declined at a similar rate ($P > 0.05$). The total content of sterols on a fresh weight basis showed no significant change under any treatment during ripening. The loss of membrane PL from was reflected by a shift in the FS to PL ratio (Table 2) ($P<0.001$). The FS/PL ratio, which increased significantly for both treatments but hardly for control, was closely correlated to the loss of Chl and increase of carotenoids contents (r=-0.64 and 0.94, respectively). Tables 3 and 4 showed the composition of the PL and FFA fractions. The PLs were rich in linolenic acid, and their ratio of PUFA to saturated FA (mol%), 2.69, was greater than that of the FFA, 2.53. Loss of PUFA from both fractions during ripening was reflected by a decrease in the ratio of PUFA to saturated FA. The decrease was greater for control than in the fruit treated with Ca ($P<0.001$).

The loss of PUFA from FFA and their low level in FFA fraction suggested LOX involvement in lipid breakdown in tomato. When analyzed, LOX specific activity has been indeed steadily increased in the control (Figure 6) ($P<0.001$). The increase was less for the fruit treated with Ca.

Discussion

The beneficial effect of Ca on the delay of ripening and senescence of some fruit and vegetables has been demonstrated [1,2]. Indeed, the loss of firmness and the preservation of quality during storage of apple and pear were often associated with a Ca deficiency. The paucity in this element is also associated with several physiological disorders such as the bitter pit in apple and the apical rot in tomato [14,36]. However, to generalize its application for other horticultural products, specific studies are necessary. In fact, the fruit and vegetables are characterized by their diversity and consequently their reactions to Ca could be different [6].

Characteristic symptoms of ripening, increase in free sugar, pH and mold development; and decrease in titratable acidity and firmness, were observed during storage of Trésor and H63-5 cultivars tomatoes at 12°C. Foliar application of $CaCl_2$ a few days before harvest caused an increase in a Ca content of the tissues and consequently influenced some of these parameters, and delayed ripening and prolonged storage life of tomatoes as demonstrated by Wills and Tirmazi [37]. Such observations were reported on strawberry, apple and pear [6,38,39].

Treatment	Days	FA				PUFA/S
		16:0	18:1	18:2	18:3	
Control	0	25.2 ± 0.8	1.9 ± 1.2	52.7 ± 1.8	13.2 ± 1.5	2.69 ± 0.03
	14	27.9 ± 1.2	5.3 ± 1.3	51.2 ± 1.3	9.6 ± 1.2	2.37 ± 0.02
	21	29.2 ± 1.0	7.2 ± 0.6	50.3 ± 1.4	7.9 ± 1.6	2.21 ± 0.02
$CaCl_2$	14	26.3 ± 1.3	3.1 ± 0.3	51.9 ± 1.6	12.7 ± 1.1	2.57 ± 0.03
	21	26.8 ± 0.9	4.9 ± 0.3	51.1 ± 1.2	9.4 ± 1.0	2.44 ± 0.02

$CaCl_2$: Dichloride Calcium; FA: Fatty Acid; PUFA/S: Polyunsaturated Fatty Acid/Sterol; RH: Relative Humidity

Table 3: FA composition (mol%) of the PL fraction of pericarp tissue during storage in darkness of tomato fruit, cv. Caruso, in a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. Values are expressed as means ± SD.

Treatment	Days	FA					PUFA/S
		16:0	18:0	18:1	18:2	18:3	
Control	0	25.9 ± 0.8	1.9 ± 1.2	3.7 ± 0.8	46.9 ± 1.5	19.6 ± 1.6	2.53 ± 0.03
	14	28.2 ± 1.2	2.6 ± 0.9	7.4 ± 1.3	50.6 ± 1.2	8.7 ± 0.9	2.17 ± 0.02
	21	30.1 ± 1.0	3.7 ± 0.6	10.9 ± 1.4	53.3 ± 1.6	6.2 ± 1.1	2.02 ± 0.03
$CaCl_2$	14	26.1 ± 1.3	–	4.2 ± 1.3	47.7 ± 1.1	14.1 ± 1.6	2.53 ± 0.02
	21	27.9 ± 0.9	2.7 ± 0.3	6.9 ± 1.2	48.4 ± 1.0	11.7 ± 0.9	2.26 ± 0.02

$CaCl_2$: Dichloride Calcium; FA: Fatty Acid; FFA: Free Fatty Acid; PUFA/S: Polyunsaturated Fatty Acid/Sterol.

Table 4: FA composition (mol%) of the FFA fraction of pericarp tissue during storage in darkness of tomato fruit, cv. Caruso, in a continuous air stream at 12°C and close to 100% RH for 21 days after foliar $CaCl_2$ application. PUFA/S. Values are expressed as means ± SD.

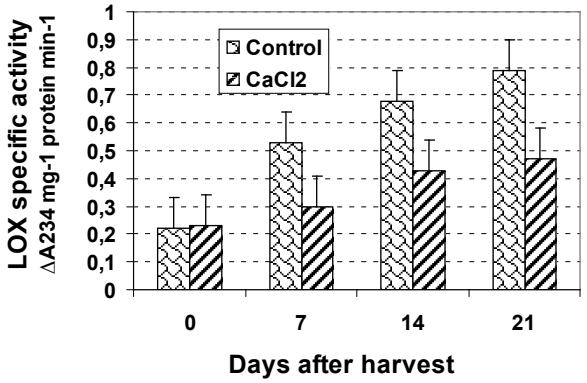

Figure 6: Change with time of LOX activity of pericarp tissue during storage in darkness of tomato fruit, cv. Caruso, in a continuous air stream at 12°C and close to 100% RH for 21 days after foliar CaCl2 application. Vertical lines show average SD for 3 replicates.

However, the response of the Ca treatment was greater for H 63-5, which contained less Ca than Trésor. The effect of Ca is less pronounced in tissues that contain adequate amounts of Ca for maintaining cell integrity [40]. The ability to accumulate and distribute Ca may vary with the cultivar and is FFinfluenced by various factors such cultivar, temperature, relative humidity, plant age, levels of other minerals in the soil (Ferguson 1984) (1). The inability of plants to accumulate and distribute Ca may partly explain why some are more prone to disorders and diseases [41].

A characteristic feature of senescence is membrane deterioration due to lipid degradation and the ensuing destabilization of the bilayer. The protection of cell membrane integrity by Ca during senescence has been explained by the ability to binding to membrane phospholipids and, in this way, to stabilize the membrane and to control membrane-associated functions [19]. Our results showed that tomato ripening, reflected by loss of Chl and increase in carotenoids, was delayed by Ca application as reported previously by Chéour et al. [17] for cabbage leaves. Lipid membrane breakdown in tomato during senescence was indicated by several markers: reduced PL content, larger ratio FS/PL and reduced rates of PUFA of the fractions PL and FFA. The levels of these markers changed in parallel with Chl degradation and carotenoids increase which are common indicators of tomato senescence. Membrane Protection from lipid degradation by Ca was explained by the decrease in most of these changes. The present results, based on the levels of intermediate products of PL breakdown, should be interpreted in the context of steady-state equilibrium between synthesis and degradation. Chéour et al. [17] have reported that the Ca can stabilize the plasmalemma by binding to the phospholipids. In fact, membrane becomes less prone to degradation by lipolytic enzymes. The undegraded lipid bilayer prevents Ca from passively entering the cytosol and facilitates the pumping of Ca outside of the cytoplasm by membrane-associated Ca^{2+}-ATPase. A low Ca level in the cytosol is essential for the normal functioning of cell metabolism. When the cytosolic Ca concentration increases, it interferes with normal biochemical activities by activating or deactivating numerous enzymes, either directly or indirectly, through various mechanisms involving a change in protein conformation, protein phosphorylation, or interaction with calmodulin [4] The constancy of the proportions of the PL classes during ripening shows that the different PLs were degraded at similar rates. The loss of PUFA from the PL classes during storage shows that the polar head-groups may have less influence on PL degradation than the FA composition of the molecular species. Such observation confirms previous reports on cabbage by Chéour et al. [17]. The marked rise in the relatively saturated FFA content, the progressive decrease in degree of unsaturation of the PL and FFA fractions, and the increasing LOX specific activity are ample evidence that LOX was involved in membrane lipid breakdown during tomato senescence. LOX (EC 1.13.11.12) is a dioxygenase that catalyzes the peroxidation of fatty acids containing a cis-cis-1, 4 pentadiene configurations [42]. Our results indicate that Ca treatment influenced its activity during tomato senescence. The decrease in the level of PUFA in the PL and FFA fractions, sign of LOX activity, was delayed by Ca treatment suggesting lower LOX activity, which was confirmed by enzyme assay.

In conclusion, foliar application led to increased Ca content of tomato fruits and delayed ripening and mol development. The response of $CaCl_2$ treatment varied with cultivar and apparently depends on the Ca content of the fruit at the time of treatment. The presence of Ca probably implies a protection of membrane lipids from degradation. Lipoxygenase, responsible for the peroxidation of the polyunsaturated fatty acids, was probably influenced by calcium in the tomato fruit.

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Dynamics of Nitrite Content in Fresh Spinach Leaves: Evidence for Nitrite Formation Caused by Microbial Nitrate Reductase Activity

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Abstract

Nitrite (NO_2^-) contained in dietary foods has long been recognized for its toxicity as the causative agent of methemoglobinemia and also as a source of mutagenic nitrosamines. Because of these potential toxicities, nitrite as well as nitrate contained in foods and drinks are strictly limited by regulations in many countries. Recent studies have offered us to update our recognition of nitrite; nitrite is an important precursor for Nitric Oxide (NO) that is required for fundamental physiological activities including vasorelaxation. Although it is well established that green vegetables contain high amounts of nitrate, there has been controversy regarding the source of nitrite accumulation in fresh green vegetables. In this study, we investigated the dynamics of nitrite and nitrate contents in spinach leaf extracts to verify the mechanisms of nitrite formation. The time course of nitrite production in leaf extracts showed a reciprocal relationship with nitrate degradation, suggesting a conversion from nitrate to nitrite. The reaction strongly depended on temperature and it was suppressed at a low temperature. Sodium tungstate, a nitrate reductase enzyme inhibitor, was effective to suppress the conversion. Pre-sterilization by autoclaving or filter sterilization completely prevented the formation of nitrite as well as degradation of nitrate. We suggest that previous reports of nitrite accumulation can be attributed to microbial nitrate reductase activities that occur during the degradation spinach leaves.

Keywords: Bacteria; Dietary food; Nitrite; Nitric oxide; Metabolic syndrome; Vegetable; Sodium tungstate

Abbreviations: AMO: Ammonia Monooxygenase; HAO: Hydroxylamine Oxidoreductase; NiR: Nitrite Reductase; NO: Nitric Oxide; NOS: Nitric Oxide Synthase; NR: Nitrate Reductase; ROS: Reactive Oxygen Species; RNS: Reactive Nitrogen Species

Introduction

A vegetable-based diet has been widely recognized effective in prevention of chronic diseases including metabolic syndrome. Nutritionally, dietary vegetable is an important source of vitamins, minerals and fibers [1]. Green leafy vegetables, in particular, are beneficial due to a high content of ascorbate (vitamin C), and colored vegetables include carotenoids (vitamin A precursor) and polyphenols (vitamin P), all of which act as strong antioxidants that detoxify Reactive Oxygen Species (ROS) formed under stress or disease conditions [2,3]. In addition to these aspects, recent progress in life science has made a new paradigm shift which impacts even on food sciences, i.e., physiology and biochemistry of Nitric Oxide (NO).

NO is a free radical gaseous molecule that was previously only recognized for its potential toxicity as an air pollutant [4]. After the discovery of a physiological function of NO in vasorelaxation mechanism, it has been revealed that NO exhibits a range of fundamental roles in mammalian physiology: regulating blood pressure [5], neurotransmission [6], regulation of immune responses through activation of macrophages [7] and penile erection [8]. Just as superoxide (O_2^-) is a primary source of ROS, NO and its reaction products are designated as Reactive Nitrogen Species (RNS) [9]. Thus, increase of NO availability through foodstuffs has attracted much attention from medical researchers in terms of chronic disease prevention [10].

In general, NO is produced in mammals through the enzymatic activity of Nitric Oxide Synthase (NOS) with the amino acid L-arginine as a substrate [5]. Since the NOS reaction requires O_2 to produce NO, it does not work in anoxia or anaerobic conditions where ischemia occurs [11,12]. Nitrite-dependent NO production mechanism does work even under such conditions [11,12], and is thereby considered to be an alternative backup mechanism for NO production in our body [11,12]. In the oral cavity, nitrite (NO_2^-) is produced from nitrate (NO_3^-) through symbiotic bacterial activities [13]. Of the nitrate absorbed from the intestine approximately one-quarter is returned to the upper gastrointestinal tract via the saliva, presumably to permit reduction of nitrate to nitrite by mouth bacterial flora [13-15].

In food science, nitrite contained in human diet has long been recognized for its toxicity as the causative agent of methemoglobinemia [12]. Due to the formation of mutagenic nitrosamines [16], it has also been presumed that nitrite is potentially carcinogenic [17]. Because of these two historical backgrounds, nitrite as well as nitrate contained in foods and drinks has been strictly limited by regulations in many countries [18-20]. However, extensive animal and epidemiological studies have not indicated that nitrite in foods leads to carcinogenesis [13].

Nitrite ingested undergoes non-enzymatic acid-mediated reduction to NO in the stomach while the rest is absorbed into the bloodstream [13]. Figure 1 shows a schematic illustration for dietary source of NO. In general, natural foods such as leafy vegetables or seaweeds possess little nitrite but high levels of nitrate [13,21] a possible health benefit of the Japanese [22] and Mediterranean diets [23-25]. Direct supply of nitrite from diets may come from processed foods or curing meats.

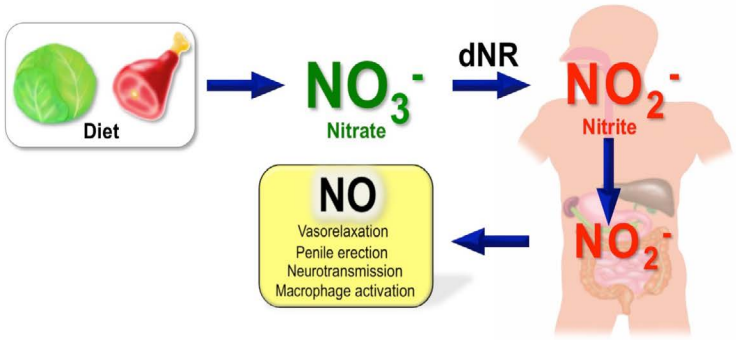

Figure 1: Exogenous sources of nitrite production in body. Dietary intakes of nitrate included in vegetables, meats and drinks lead to conversion of nitrate to nitrite by nitrate reductase of oral bacteria. Nitrite reaching the stomach through the digestive tract can be spontaneously converted to nitric oxide (NO) in the acidic conditions of the stomach. NO acts as an important factor in physiological functions, for example vasorelaxation, neurotransmission and activation of macrophages. dNR indicates dissimilatory nitrate reductase.

Therefore, bioavailable nitrite is mostly supplied exogenously as nitrate contained in foods and drinks (Figure 1).

Vegetables are major dietary sources of nitrate in general. More than 80% of the nitrate ingested can be attributed to vegetables [26]. Nitrate is an essential nutrient for plant growth [27] and its content varies widely between plant species [28,29] and even within the same tissue types of the same species, presumably due to differences in nitrate fertilization during cultivation [30]. Even in plant physiology, the conditions where leaves accumulate nitrite in the tissue are yet unclear [31]. Many physiological studies have suggested that plants produce nitrite only under stress conditions [32,33]. However, its production mechanism is still under debate [34].

There have been many reports on the nitrate and nitrite contents in retail green vegetables [28,35]. In respect to nitrite content, there are inconsistent and controversial results; some reports indicate the presence of nitrite [28,35-37] but others do not [38,39]. Hsu et al. suggested that nitrite detected in fresh vegetables may be due to bacterial activities [29]. To date, however, there has been no experimental evidence for the bacterial association with the nitrite formation. Here we demonstrate that the nitrite formation can be attributed to bacterial nitrate reductase activity which converts nitrate to nitrite in the process of spinach leaf decay.

Materials and Methods

Sample preparation

Spinach (*Spinacia oleracea* L.) was used as a representative green leaf vegetable due to a high amount of nitrate [40]. Fresh spinach leaves were obtained from local markets. After removing midrib, spinach leaves washed with tap water (300 g of fresh weight) were homogenized with distilled water (600 ml) at 0˚C for 30 s. The obtained homogenate was filtered through four-layers of gauze. The filtrate was centrifuged at 5,000 × g for 12 min and the supernatant obtained was used as the leaf extract for experiments.

The leaf extract (10 ml) was incubated in a gamma-sterilized Falcon tube at a constant temperature in darkness. During incubation, the tubes were continuously shaken at 120 rpm. Samples collected were frozen at –80˚C until analyzed.

The frozen samples were thawed and denatured at 100˚C for 3 min to inactivate endogenous enzymatic activities. The heat-denatured samples were centrifuged at 15,000 × g for 10 min (4˚C) and the supernatant filtered with a syringe filter (0.45 µm mixed cellulose ester syringe filter, 25AS045AN, Advantec) to obtain a soluble fraction. We further passed the sample through a SPE removing chloride filter (IC-Ag, Altech) to remove Cl^- whose retention time was close to nitrite. A 0.2 µm PTFE syringe filter (13HP020CN, Advantec) was used before injection into the ion chromatography apparatus.

Ion chromatography

To quantify the nitrate and nitrite contents, we used HPLC ion chromatography with an anion column (Shim-pack IC-A3, Shimadzu) along with an electrical conductivity detector (CDD-10A_{VP}, Shimadzu). Experimental conditions were basically similar to the method reported by Ogata and coworkers [41]. A mobile phase liquid contained 3.2 mM Bis-tris, 8.0 mM 4-hydroxybenzoic acid and 50 mM boric acid (pH 4.5). The flow rate was 0.7 ml/min and the column temperature was kept at 40˚C. Calibration was carried out with an anion mixture standard solution (Wako, Saitama).

Time course experiments to monitor nitrite and nitrate contents

For the time-course experiments of changes in nitrate and nitrite contents, 200 ml of leaf extract in a 500 ml conical flask was used. Conical flasks were plugged with sponge silicone plugs which allow gas flow but prevent microbe penetration. The leaf extracts were incubated in darkness at 35, 25 and 15˚C for 48 h. During the incubation, conical flasks were rotated at a constant speed (60 rpm) for mixing and aeration. Aliquots (1 ml) of the leaf extract were taken and they were frozen at –20˚C until analyzed.

Experiments of inhibitors and sterilization

To verify microbial activities we sterilized the leaf extract with two distinct methods: autoclave sterilization and filter sterilization. Autoclaving was carried out at 120˚C for 5 min before the incubation. To sterilize without heat treatment, we used a filter sterilization method with a 0.22 µm PES filter cartridge (8020-500, IWAKI) before the incubation. In inhibitor experiments, allylthiourea, nitrapyrin and tungstate (1 mM for each) were added before incubation, and the leaf extracts (10 ml) were rotated at 120 rpm at 25˚C in darkness for 42 h. Aliquots of leaf extract (1 ml) were sampled and kept at –80˚C until analyzed.

Chemicals

Allylthiourea (1-allyl-2-thiourea) and nitrapyrin (2-chloro-6-

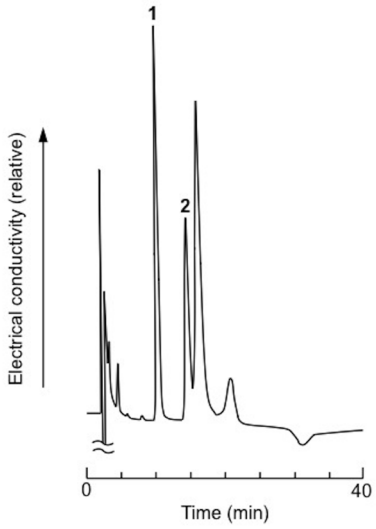

Figure 2: Ion chromatogram of spinach leaf extract. A typical chromatogram is shown. Peak 1, NO_2^-. Peak 2, NO_3^-. Each peak area was quantified with the anion mixture standard solution.

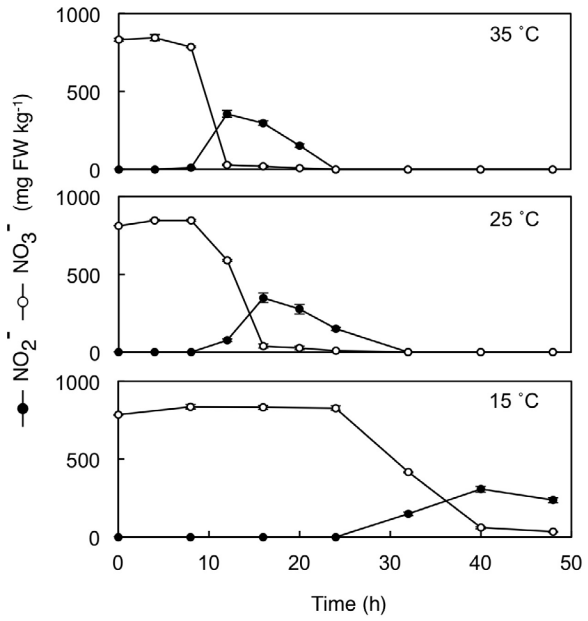

Figure 3: Dynamics of nitrate and nitrite. Closed circle, NO_2^-. Open circle, NO_3^-. Spinach leaf extracts were incubated at 35˚C, 25˚C and 15˚C in darkness. Each point represents the average with error bars (S.E., n=3).

[trichloromethyl] pyridine) were obtained from Tokyo chemical. Tungstate (sodium tungstate dehydrate) was obtained from Nacalai tesque. Bis-tris was obtained from Dojindo. 4-Hydroxylbenzoic acid was obtained from Kishida chemical. Allylthiourea and tungstate were dissolved in distilled water (100 mM) and nitrapyrin was dissolved in DMSO (100 mM).

Results

Nitrate content in spinach leaves

As many studies have reported so far, the nitrate content in retail spinach leaves varies to a large extent: from a minimum of 110 mg/kg [37] and a maximum of 4,923 mg/kg [35]. We also found similar trend on the nitrate content in retail spinach leaves. In our analysis, the average nitrate content was 1,900 mg/kg ±194 (n=10; ±SD), with a 785 mg/kg as minimum and maximum of 2,544 mg/kg.

Although our results of nitrate content basically agreed with the previously reported values, we detected only negligible amounts of nitrite. As Phillips pointed out, some previously reported results might be accounted for by bacterial nitrite formation due to decay of vegetables [42]. We indeed found that nitrite was detected in one week artificially decayed spinach leaves (data not shown). Since experimental control of the decay of the leaves with good reproducibility was difficult and it was virtually impossible to apply chemicals to investigate the phenomenon, we decided to use a model system for analysis. In this study a crude leaf extract that includes a soluble fraction as well as small organelles and surface bacteria was used for analysis.

Time course of dynamics of nitrate and nitrite at different temperature

We first monitored the changes in nitrite and nitrate contents in spinach leaf extracts for 48 h, incubating at different temperatures. To quantify nitrate and nitrite, we applied an ion chromatography technique. Figure 2 shows a typical HPLC ion chromatogram. Nitrate (peak 1) and nitrite (peak 2) were successfully detected as distinct peaks (Figure 2). Nitrate and nitrite contents were determined with each peak area. Figure 3 illustrates changes in nitrate and nitrite contents incubated at 35˚C, 25˚C, and 15˚C. At all the temperatures tested, nitrate degradation followed by nitrite formation was observed during the incubation (Figure 3). The changes strongly depended on an incubation temperature; both changes (nitrate degradation and nitrite formation) went slower at a lower temperature. At 15˚C, no change was observed until 24 h. The maximum nitrite content was observed among the all incubation temperatures tested: 12 h (35˚C), 16 h (25˚C) and 40 h (15˚C). The maximum values of the formed nitrite at the peaks were 357 mg/kg, 350 mg/kg, and 307 mg/kg at 35˚C, 25˚C, and 15˚C, respectively. After reaching to the maximum, nitrite content decreased and eventually reached a negligible amount at all temperatures. It should be noted that an odd smell came from such leaf extracts.

Effects of inhibitors of bacterial nitrification and denitrification

To verify the involvement of biological activity in nitrite formation in the leaf extract, we investigated effects of inhibitors on nitrite formation. Inhibitors of nitrification (allylthiourea and nitrapyrin) and denitrification (tungstate) were tested.

Figure 4 shows suppressive effects of various treatments on nitrite formation in spinach leaf extract. As a negative control, we analyzed sterilized leaf-extract samples: autoclaved or filter sterilized. In both sterilization methods (with or without heat treatment), no nitrite formation was observed, indicating that the nitrite formation is attributable to microbial activities. Allylthiourea and nitrapyrin are known to inhibit the bacterial Ammonia Monooxygenase (AMO) which is a key enzyme for nitrification [43]. Allylthiourea showed no effect while nitrapyrin exhibited some (20% suppression). Tungstate is an inhibitor for nitrate reductase which is involved in denitrification. Interestingly, tungstate effectively prevented nitrite formation even in non-sterilized leaf extract sample (Figure 4).

The same trend was observed in the degradation of nitrate in the leaf-extract (Figure 5). As observed in nitrite formation, sterilization by autoclave and filter prevented the degradation of nitrate contained

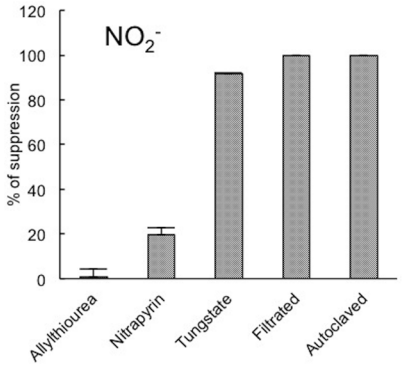

Figure 4: Suppression of nitrite formation. Inhibitory effects of enzyme inhibitors allylthiourea (AMO inhibitor), nitrapyrin (AMO inhibitor) and tungstate (nitrate reductase inhibitor) on NO_2^- formation activity. The final concentration was 1 mM for each. To confirm the association of microbial activities, the leaf extracts were treated by either filter or autoclave sterilization. Each bar represents the average with error bars (S.E., n=3).

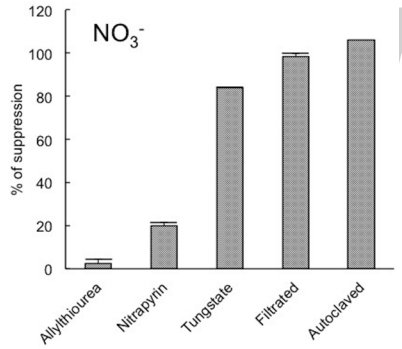

Figure 5: Suppression of nitrate degradation. Inhibitory effects of enzyme inhibitors allylthiourea (AMO inhibitor), nitrapyrin (AMO inhibitor) and tungstate (nitrate reductase inhibitor) on degradation activity of NO_3^-. The final concentration was 1 mM for each. To confirm the association of microbial activities, the leaf extracts were treated by either filter or autoclave sterilization. Each bar represents the average with error bars (S.E., n=3).

in the leaf-extract (Figure 5). Tungstate effectively prevented the degradation of nitrate, whereas nitrapyrin and allylthiourea showed weaker effects. Overall, the effects of the treatments were identical between nitrite formation and nitrate degradation, suggesting that bacterial conversion of nitrate to nitrite occurred in the leaf extract as has been suggested.

Discussion

Fresh spinach leaves do not accumulate nitrite

Our results have provided experimental evidence to confirm that nitrite accumulation merely occurs in fresh intact vegetables; bacterial activity mediates nitrite formation through nitrate reducing activity in decayed leaves. This nitrite formation can be suppressed at a low temperature, implying that nitrite should not be accumulated as long as fresh intact spinach is properly stored in a refrigerator at a non-frozen temperature. Therefore, it can be concluded that previous reports of high nitrite content reflected bacterial activity due to physical damage or degradation of spinach leaves as discussed by Phillips [42].

Nitrite accumulation mechanism in spinach leaves

As mentioned in the introduction, plant leaves do not accumulate nitrite under favorable conditions; nitrite can be detected in leaves when plants are exposed to stress conditions. Nitrite exists as an intermediate metabolite in nitrate assimilation. Plants absorb nitrate mainly from soil to synthesize amino acids. Using solar light energy, absorbed nitrate from the soil is reduced to nitrite by Nitrate Reductase (NR) in the cytosol [4]. Nitrite translocated into the chloroplasts is converted to ammonium ion (NH_4^+) by nitrite reductase (NiR) located in the chloroplasts [4] and then ammonium ion is assimilated into amino acids [4]. The process can be disturbed by biotic (infectious or herbivoric) as well as abiotic (environmental) stresses. It has been found that root of tomato (*Lycopersicon esculentum* cv. Rondello) forms nitrite under anoxic conditions [33]. In rice seedling (*Oryza sativa* L. cv. Akitakomachi) Suzuki et al. reported that exposure of the tissues (root and stem) to different temperatures resulted in accumulation of nitrite in the leaves after light/dark transition [32]. It should be emphasized that accumulated nitrite can be reduced to NO by NR and the gas will be released into the air [4,44]. At an early stage of this study, we hypothesized that retail spinach leaves stored at a refrigerator temperature under light for display might accumulate nitrite because ROS is overproduced under such condition [45]. We did detect a small and transient NO emission from leaves but nitrite content was negligible (data not shown). As long as we examined, there was no clear indication for the accumulation of nitrite in spinach leaves by stress treatment that is presumed to occur in a retail process. The only case we detected nitrite was following physical damage of the leaves, which facilitated bacterial degradation.

It has been known that processed vegetable foods sometimes include high nitrite. Chetty and Prasad; and Phillips reported that baby foods made from vegetable included nitrite [42,46]. Moreover, some studies reported nitrite in canned and frozen vegetables [37,42,47]. It is highly likely that vegetables were already spoiled, and sterilization was not enough to prevent microbial growth in the processing vegetables. Fermentation in pickling vegetable can result in high accumulations of nitrite. Ji et al. reported that nitrite was formed and nitrate was degraded in pickled Chinese cabbage (*Brassica campestris* L.) during the fermentation [48]. Yan et al. found that nitrite in cabbage (*Brassica oleracea* var. capitata) increased during fermentation for Chinese paocai [49].

Another reason for inconsistency in nitrite content in vegetables may be attributed to artefactual factors due to the methods used. Many earlier studies used the Griess method (a colormetric method) for nitrite quantification of vegetables. The tissues of plants or vegetables contain abundant antioxidants or reductants such as ascorbate and polyphenols which may affect certain assays [3]. Additionally, photosynthetic pigments may act as photosensitizers to produce reactive oxidants such as ROS under light [4]. These technical factors might confound the quantification of nitrite.

Source of bacteria

In principle, nitrite is formed through two distinct dissimilation metabolisms, namely, nitrification and denitrification [31,50]. In nitrification, two enzymes, Ammonium Monooxygenase (AMO) and Hydroxylamine Oxidoreductase (HAO), are involved in nitrite formation [31,50]. Ammonium ion is oxidized to hydroxylamine by AMO. Subsequently, HAO converts hydroxylamine to nitrite [31,50]. In denitrification, nitrite is formed from nitrate by nitrate reductase similar to plants [31,50]. Allylthiourea specifically inhibits AMO by binding to copper in its active center [43,51], whereas nitrapyrin inhibits AMO by acting as an alternative substrate [43]. Protein modification by the oxidized product irreversibly inactivates not only AMO but other

proteins in the cell as well [43,52]. Tungstate competes with molybdate in molybdate-dependent enzymes including NR, resulting inactivation of these enzymes [53,54]. In this study, we examined the effects of inhibitors of nitrification or denitrification on nitrite formation and nitrate degradation. As shown in Figures 4 and 5, nitrite formation and nitrate degradation were strongly inhibited by tungstate, whereas that allylthiourea and nitrapyrin had little effects. Based on these results, it is reasonable to conclude that nitrite formation was due to denitrification by NR but not nitrification by AMO and HAO. It should be noted that bacterial populations colonize in spermosphere as well as phyllosphere of spinach (*Spinacia oleracea*) seedlings and plants [55]. The nitrifying bacteria that produce nitrite would be included in such spermosphere and phyllosphere microbiota.

Prospects

Nitrite has long been considered as a toxic agent in human diet. Since inorganic nitrate is not an essential nutrient for humans, intake of nitrate and nitrite has been thought to be nonbeneficial. However, recent studies have suggested that dietary nitrate and nitrite are important for preventing metabolic syndromes [10]. In fact, there are less cardiovascular diseases in regions where consumption of vegetables is high [56] whereas there is no strong evidence that nitrate and nitrite intake from foods results in methemoglobinemia [57]. This is probably because, unlike infants, adults can reduce methemoglobin by the activity of NADH-cytochrome b5 reductase [58]. Also, vegetables contain lots of antioxidants as well as molecules that can suppress nitrosative stress. We suggest that nitrate contained in vegetables is beneficial for human health and that nitrite is not contained at physiologically effective concentrations in leafy vegetables as long as they are properly stored.

Acknowledgements

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Nutrition and Waist Circumference as an Indicator of Abdominal Obesity in 17-18-Year-Old Youth

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Abstract

Proper nutrition is one of the most important environmental factors that affect the development of the young organism as well as the maintenance of good health in adulthood. The harmonious development of the young organism can be disrupted by both excess and deficiency of nutrients and by misbalance between them. The aim of this study was to assess the impact of diet on the waist circumference being an indicator of abdominal obesity in 17-18-year-old youth from Wroclaw and vicinity.

The survey was conducted in selected secondary schools in Wroclaw that participated in the «Health-Promoting School» program in the period from November 2010 to May 2011. Quantitative nutritional survey was performed using the direct 24- hr dietary recall method repeated seven times. Calculations of the content of each nutrient and energy supply in the daily diet of surveyed students were made using the «Energy» V.4.1. software with a database created based on «Table of Composition and Nutritional Value of Food Products» by Kunachowicz et al.

Abdominal obesity was found among 25.6% of girls and 15.6% of boys aged 17-18 years. Above-normal waist circumference was observed more frequently among the 18-year-old youth, and the differences were statistically significant. There was a statistically significant difference in energy and macronutrient intake that was dependent on both gender and particular percentile group. The supply of energy, compared with the relevant dietary guidelines (EAR), was too low for girls and boys, regardless of waist circumference, which was also, affected by the poor implementation of dietary allowances (RDA) for nutrients, especially for total carbohydrates, fiber and plant protein in daily meals. Meals of girls and boys with the smallest waist circumference provided energy and nutrients in amounts close to the relevant allowances (RDA), as opposed to diet of the surveyed with the waist above the 95th percentile.

The coverage of norms for energy (EAR) and macronutrients (RDA) was closest to normal in the adolescents with the smallest waist circumference.

Keywords: Youth; Eating habits; Daily food ration; Nutritional risk factors; Abdominal obesity

Introduction

Proper nutrition is one of the most important environmental factors that affect the development of the young organism as well as the maintenance of good health in adulthood. The harmonious development of the young organism can be disrupted by both excess and deficiency of nutrients and by misbalance between them. A well-balanced daily food ration for youth should provide adequate energy and optimum amounts of essential nutrients, vitamins and minerals [1].

Epidemiological surveys conducted around the world show dietary faults in a large group of children and young people. They may include the following: inadequate number of meals throughout the day, lack of regularity and prolonged intervals between meals, mealtimes shifted to the late hr of the evening and meal replacement with fast food [2,3].

Dietary risk factors that significantly affect the incidence of obesity among children and youth include: inadequate intake of energy and nutrients in daily food ration (DFR) and very low level of physical activity.

Obesity is defined as an excessive accumulation of body fat, leading to impaired function of the human body, which is responsible for increased morbidity and mortality [4]. Abnormal waist circumference is the most important indicator of visceral fat mass, which plays an important role in the pathophysiology of insulin resistance, hemodynamic changes, and lipid and carbohydrate metabolism.

The report of British Medical Association Board of Science [5] shows that 155 million children and young people worldwide are overweight or obese. Globally, the phenomenon of overweight and obesity in children and youth continues to increase, reaching in the general population and in the Western world, proportions of pandemic of the 21st century. The annual increase in global obesity incidence in 1970s was 0.2%, in the 1980s it was 0.6%, and in 2000 it already reached 2% [6]. Only in the European countries, the problem of overweight concerns 14 million children and youth, increasing within each year by further 400,000. Obesity Task Force (IOTF) [7] estimates that 1 in 5 children in Europe are overweight. In Poland in the last 20 years, the percentage of obese children and youth has been successively increasing from 2.5 to 20% of the population [8].

On the one hand, the increasing incidence of overweight and obesity among children and youth, as a major risk factor for a number of food-related diseases, is concerning, but on the other hand, an excessive body mass control and the desire to reduce the weight in the same population, is also worrying. The adherence to restrictive eating

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behaviors to achieve lean body size is caused by many factors, including social and psychological reasons [6,9].

The aim of this study was to assess the impact of diet on the waist circumference being an indicator of abdominal obesity in 17-18-year-old youth.

Materials and Methods

Characteristic of the study group

The survey was conducted in selected secondary schools in Wroclaw that participated in the «Health-Promoting School» program in the period from November 2010 to May 2011. In the group of 17-18 year old (n=269) students, girls accounted for 59.5% and boys constituted 40.5%. 72.9% of participants were from Wroclaw, 11.1% lived in towns, while 15.2% in rural areas. The largest group (69.9%) in the surveyed population was this originating from complete families-living only with their parents or with additional siblings. 30.1% of young people came from incomplete families.

Exclusion criteria from the study group were: age over 18 years, lack of permission from parents and young people to participate in the study, and incomplete documentation.

Nutritional survey

Quantitative nutritional survey was performed using the direct 24 hr dietary recall method repeated seven times. To determine the size of food rations consumed, «Photographic Album of Food Products and Dishes» was used, which was developed at the National Institute of Food and Nutrition (IZZ) [10].

Calculations of the content of each nutrient and energy supply in the daily diet of surveyed students were made using the «Energy» V.4.1. Software with a database created based on «Table of Composition and Nutritional Value of Food Products» by Kunachowicz et al. [11]. To compare the results with the current dietary guidelines [12] for estimated average requirements (EAR), a moderate physical activity was adopted in the test group and the mean body mass of 56 kg for girls and 67 kg for boys.

In the qualitative assessment of nutritional determinants, a questionnaire was used that was developed at the Department of Human Nutrition, Wroclaw University of Environmental and Life Sciences. Socioeconomic questionnaire was completed individually and included questions about the qualitative assessment of diet including the number, frequency and location of meals consumption throughout the day.

Anthropometric measurements

The following measurements have been made among the young people in the study: body mass, body height, waist circumference and hip circumference. Measurements of body mass and height were taken on an empty stomach in the morning, using medical scales with stadiometer, without shoes and upper clothing. Measurements of waist circumference and hip circumference were performed using an anthropometric tape.

Centile charts developed at the Children's Memorial Health Institute [13] were used to evaluate the anthropometric parameters. The following cut-off points were considered: waist circumference >95th percentile - abdominal obesity (central) (17-18-year-old girls >80 cm, 17-year-old boys >90 cm, 18-year-old boys >91 cm), BMI >85th percentile-overweight (17-18-year-old girls 23.9-26.6 kg/m^2, 17-year-old boys 24.9-27.4 kg/m^2, 18-year-old boys 25.5-28.2 kg/m^2), BMI index >95th percentile - obesity, 17-18-year-old girls >26.6 kg/m^2,17-year-old boys >27.5 kg/m^2, 18-year-old boys >28.2 kg/m^2.

Statistical analysis

All results were subjected to statistical analysis using Stat Soft software STATISTICA 10. In order to characterize the study group of young people, median (Me), quartile deviation (Q), and the 75th and 95th percentile values were calculated for selected anthropometric parameters. Accordance of continuous anthropometric and nutritional data with normal distribution was evaluated with the Shapiro-Wilk test. In the absence of consistency with the normal distribution, the differences in the supply of energy and essential nutrients, depending on the subgroup defined by waist size, were assessed by using the following non-parametric tests: the *Kruskal-Wallis* test (for comparisons between three groups) or the *Mann-Withney U* test (for comparisons between groups of girls and boys), with a statistical significance level at $p<0.05$.

Significant correlations between discontinuous dietary factors and selected anthropometric parameters were calculated using multi-way tables and the correlations between them were calculated with the Chi^2 test.

Results and Discussion

The surveyed adolescents were divided into three subgroups according to waist circumference:

- Subgroup 1 (n=131, Girls=62, Boys=69), waist circumference <75th percentile
- Subgroup 2 (n=80, Girls=57, Boys=23), waist circumference 75-95th percentile
- Subgroup 3 (n=58, Girls=41, Boys=17), waist circumference <95th percentile

The occurrence of obesity among the young people in the subgroups of boys and girls by age is shown in Figure 1.

Abdominal obesity was found among 25.6% of girls and 15.6% of boys aged 17-18 years (Figure 1). Above-normal waist circumference was observed more frequently among the 18-year-old youth, and the differences were statistically significant.

Observations made by Banas [14] in 325 children aged 7-16 years showed abdominal obesity in 14.6% of girls and 20.2% of boys. The prevalence of abdominal obesity, as in own study, was higher among older children [14]. In turn, the study of Tremblay et al. [15] involving 2879 children aged 7-13 years, showed the occurrence of obesity among 10% of boys and almost 9% of girls aged 7-13 years [15]. Study carried out in the United States has shown that the prevalence of obesity among over 4,000 children and adolescents aged 2-19 year, participating in the NHANES project, was 16.9% [16].

Improper diet, increasing the risk of food-related diseases, was mainly due to unbalanced supply of energy and nutrients, and abnormal number of meals eaten in a day. Table 1 shows the energy intake and macronutrients' composition in the food rations of the young people.

There was a statistically significant difference in energy and macronutrient intake that was dependent on both gender and particular percentile group. The supply of energy, compared with the relevant dietary guidelines (EAR), was too low for girls and boys, regardless of waist circumference, which was also, affected by the poor implementation of dietary allowances (RDA) for nutrients, especially

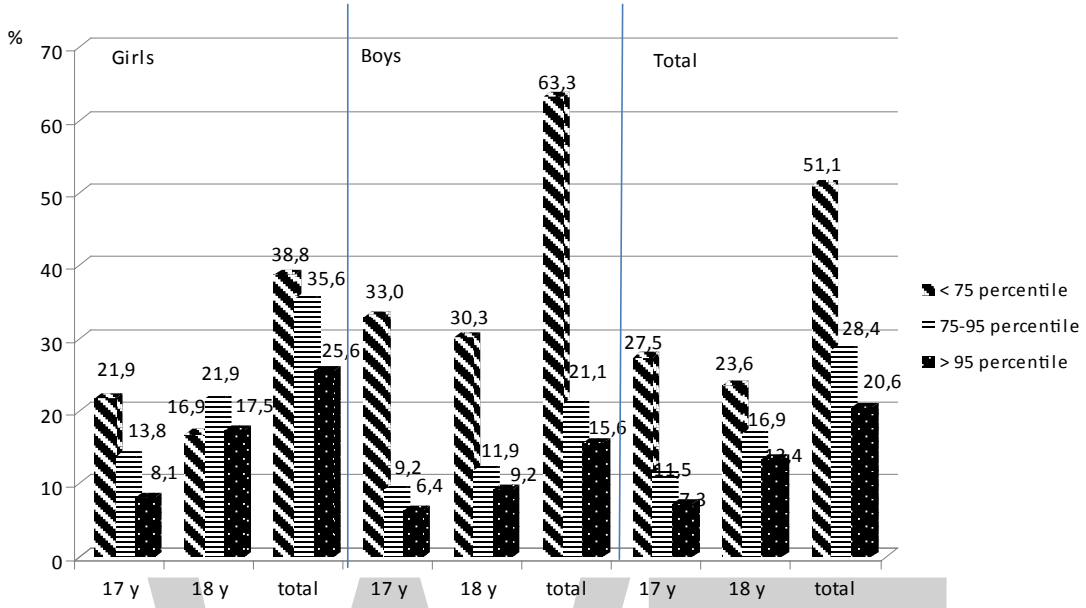

Figure 1: The occurrence of obesity among the young people in the subgroups of boys and girls by age (n=269).

	Waist circumference [cm]	Energy [kcal]	% of norm (EAR) coverage	Total protein [g]	% of norm (RDA) coverage	Animal protein [g]	% of norm (RDA) coverage	Plant protein [g]	% of norm (RDA) coverage	Total fat [g]	% of norm (RDA) implementation	Carbo-hydrates [g]	% of norm (RDA) coverage	Choleste-rol [mg]	% of norm (RDA) coverage	Fiber [g]	% of norm (RDA) implementation
Girls n=160	< 75 pc n=62	1445.0 ± 307.5*	56.7#	51.4 ± 11.0*	124.5	33.9 ± 9.2*	158.6#	17.3 ± 3.6*	84.9#	56.0 ± 14.2*	67.5#	189.8 ± 31.4*	47.5#	167.4 ± 51.0	55.8#	10.6 ± 3.1	50.2#
	75-95 pc n=57	1417.5 ± 272.5	57.8	48.0 ± 8.4	109.5	28.5 ± 6.0	131.8$	16.9 ± 3.6	81.3	52.6 ± 14.6	63.4	194.9 ± 41.6	50.1	156.4 ± 56.8	52.1$	10.5 ± 2.8	50.0$
	> 95 pc n=41	1181.0 ± 335.5*	47.2	42.1 ± 7.0*	68.8^	24.9 ± 5.2*	83.6^	16.3 ± 2.9*	51.8	47.9 ± 17.4*	57.7	159.7 ± 42.5*	42.1	149.1 ± 45.3	49.7^	9.4 ± 2.5	44.8*
	Total n=160	1384.5 ± 304.0	55.4	47.7 ± 9.6	100.8&	29.5 ± 7.2	123.9&	16.9 ± 3.4	75.1&	52.8 ± 16.3	63.5&	82.7 ± 41.4	46.4&	161.4 ± 48.6	53.8&	10.4 ± 2.9	49.5&
Boys n=109	< 75 pc n=69	2312.0 ± 512.0*	68.0#	83.7 ± 19.4*	157.8	54.3 ± 13.5*	199.1#	29.1 ± 7.1*	114.0#	90.7 ± 26.3*	80.3#	308.9 ± 60.8*	56.5#	309.3 ± 79.1	103.1#	17.4 ± 3.9*	82.4#
	75-95 pc n=23	2267.0 ± 601.0	66.7	72.8 ± 17.2	113.9	43.0 ± 13.1	145.8$	25.2 ± 7.6	83.2	87.4 ± 25.9	77.3	245.3 ± 643*	45.8	292.5 ± 102.0	97.5$	12.7 ± 4.0	60.5$
	> 95 pc n=17	1992.0 ± 345.5*	58.6	71.1 ± 13.9*	92.5^	43.4 ± 10.5*	122.0^	24.4 ± 3.8*	65.3	74.1 ± 13.2*	65.6	258.9 ± 25.2	49.9	265.4 ± 82.6	88.5^	12.7 ± 1.4*	65.5*
	Total n=109	2267.0 ± 458.0	66.7	74.3 ± 18.9	140.4&	47.9 ± 13.2	178.8&	21.6 ± 6.9	97.7&	87.4 ± 21.4	77.3&	287.7 ± 58.6	53.9&	298.4 ± 82.2	99.5&	15.8 ± 3.8	75.2&

* - Statistically significant differences between subgroups within gender (Kruskal-Wallis test) #, $, ^, & - Statistically significant differences between genders (Mann-Whitney U test)

Table 1: The supply of energy and macronutrients in food rations of the young people by gender and waist circumference (n = 269).

for total carbohydrates, fiber and plant protein in daily meals (Table 1). Meals of girls and boys with the smallest waist circumference provided energy and nutrients in amounts close to the relevant allowances (RDA), as opposed to diet of the surveyed with the waist above the 95th percentile.

The study of Piotrowska and others [17], conducted among 409 girls aged 16-18 years from Wroclaw secondary schools, showed that diets of teenagers were meeting 78% of recommended intake for energy (EAR). This result was higher than in own study. Greater coverage of recommended allowances (RDA) was achieved in the case of all macronutrients: proteins were at the level of 136% of the norm, fat at 90%, and carbohydrates at 65% of the norm. Fiber intake was at a similar level as in the present study and amounted to 16.9 g/day/person [17].

The study of Falkowska et al. [18], in 866 children aged 10-12 years, has shown that meals of girls and boys who are overweight or obese, met the norms for energy (EAR) and macronutrients (RDA) to a greater extent than meals of children with normal weight. The coverage of the norm for energy among people with excess body mass was 111.5% in girls and 114.1% in boys. The differences in energy supply between subjects with normal and excessive body weight were statistically significant within gender [18].

A study of 512 girls and boys aged 11-14 years in Greece showed that the intake of energy and macronutrients in daily meals was higher than in our study, both for children with normal body mass and for those with overweight and obesity [19]. Nevertheless, the intake of proteins, fats and carbohydrates by Greek girls with overweight and obesity, was significantly lower than by Greek boys with excess body mass [19]. (Table 1) (Figure 2).

The number of meals consumed by the surveyed young people during the day is shown in Figure 2. We have shown that three meals a day were consumed by 21.1% of the young people with waist circumference below the 75th percentile, 15% of those with waist circumference between the 75-95th percentile and 12.6% of youth with the largest waist circumference. Own study showed no significant differences in the number of meals consumed between girls and boys (Figure 2). However, significant differences were found between girls and boys with waist circumference below the 75th percentile and between the 75 and 95th percentile, in the frequency of eating four or more meals a day. Significant differences were also demonstrated between girls and boys with waist circumference between the 75 and 95th percentile and above the 95th percentile, in the frequency of eating three meals. It was also found that significantly younger people with waist circumference below the 75th percentile consumed three meals and four or more meals a day.

The study of Gajda and Jezewska-Zychowicz [20] showed that more than half (54.5%) of the 185 surveyed secondary school students ate 4-5 meals a day, and about one third declared consumption of three or fewer meals per day. There were no significant differences between girls and boys. The study of Wajszczyk et al. [21] also showed no significant differences in the number of meals consumed by girls and boys. About 45% of young people from 86 classes in Warsaw elementary and secondary schools eaten four meals a day, and less than 21% declared eating three meals a day. The study of Piotrowska and others [22] reported that among 409 female teenagers from Wroclaw aged 16-18 years; significantly more girls consumed 4-5 meals per day that assessed their own body weight as «just right» than those who thought they were «too fat». It also showed a statistically significant positive correlation between the number of meals and satisfaction with owns appearance [22]. (Table 2)

The present study determined the frequency of consumption of all-day meals by the surveyed adolescents (Table 2). First breakfast was eaten every day by 23.1% of girls with waist circumference below the 75th percentile, and only by 14.4% of girls with the largest waist

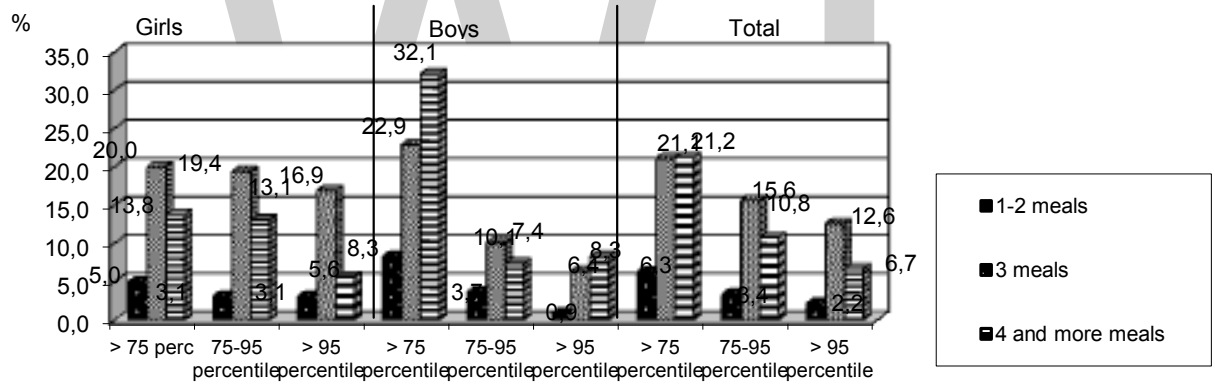

Figure 2: Number of meals eaten by the surveyed youth during the day, in the subgroups of girls and boys divided by waist circumference (n=269).

Waist circumference [pc]	Surveyed group	1st breakfast		2nd breakfast		Dinner		Afternoon snack		Supper		Snacking	
		every day [%]	never [%]	every day [%]	never [%]	every day [%]	never [%]	every day [%]	never [%]	every day [%]	never [%]	every day [%]	never [%]
<75 pc	Girls n=62	23.1#	1.9	15.6	10.0	31.3	0.0	8.8	13.1	23.9	2.5	20.0	3.1
	Boys n=69	41.3^	8.3	25.7	17.4	59.6	0.6	13.8	22.0	49.1	0.0	33.9	8.3
	Total n=131	30.5*	4.5	19.7	13.0	42.3	0.0	10.8	16.7	34.1	1.5	25.7	5.2
75-95 pc	Girls n=57	19.4	5.6	16.3	8.1	31.3	0.6	7.5	12.5	23.3	3.1	22.5	1.9
	Boys n=23	8.3^	1.8	8.3	3.7	17.4	8.1	8.1	10.1	15.7	1.9	4.9	6.7
	Total n=80	14.9*	4.1	13.4	6.3	25.7	0.4	5.2	11.5	20.2	2.6	15.2	3.7
> 95 pc	Girls n=41	14.4#	3.8	8.8	10.0	18.1	0.0	1.9	14.4	12.0	1.9	6.9	5.8
	Boys n=17	13.8	0.0	5.5	2.8	14.7	0.0	0.9	6.4	11.1	0.0	6.4	5.5
	Total n=58	14.1*	2.2	7.4	7.1	16.7	0.0	1.5	11.2	11.6	1.1	6.7	5.2

*,#,^, $, @, +, ! - statistically significant difference (Chi^2).

Table 2: Consumption of meals in the studied group divided by waist circumference and gender (n=269).

circumference. Among boys, eating 1st breakfast daily was declared by 41.3% with the smallest waist circumference, and only by 13.8% of boys with waist circumference above the 95th percentile. Everyday snacking between meals was declared by 25.7% of young people with the smallest waist circumference, and by 6.7% of the group with waist circumference above the 95th percentile. There was a statistically significant difference observed between the percentile subgroups in the frequency of eating 1st breakfast. Among both girls and boys, 1st breakfast was consumed significantly more often by these with the smallest waist circumference. Girls with waist circumference between 75 and 95th percentile and boys with waist circumference below the 75th percentile significantly more often declared not to eat breakfast. Girls did not eat breakfast more often than boys. Not eating 2nd breakfast was also significantly more often declared by girls than by boys, and this was recorded regardless of waist circumference. Dinner was consumed significantly more often by boys and girls with waist circumference smaller than the 95th percentile. Snacking was significantly more often reported by girls, and least often by girls with the largest waist circumference.

The study of Malary et al. [23] showed that 30% of 309 surveyed teens from Silesia voivodeship did not eat 1st breakfast, and about 16% declared not to eat dinner, which was more often recorded for girls than boys. The same held true for the consumption of dinner-72% of lower secondary school female students did not eat dinner in comparison to female students from upper secondary school where about 40% declared not to eat supper. Almost 70% of all subjects were regularly snacking between meals. Among the 409 female teenagers studied by Piotrowska et al. [22], having 1st breakfast every day was declared by 51.6% of the group, whereas no breakfast was declared by 15.2%. Regular consumption of meals was significantly associated with maternal education. Girls whose mothers had a secondary education ate meals with higher regularity than those whose mothers had vocational education. The study of Gajda [20] found that ¼ of the surveyed population almost never consumed 1st breakfast, nearly 5% did not eat dinners, and almost 10% did not eat suppers. Regular snacking was declared by 35.5% of respondents. There were no statistically significant differences in the frequency of eating between boys and girls.

Conclusions

- The coverage of norms for energy (EAR) and macronutrients (RDA) was closest to normal in the adolescents with the smallest waist circumference.
- The habit of eating 1st breakfast was found significantly more often in young people with the smallest waist circumference.
- Regular eating was more common among youth with waist circumference below the 75th percentile than among other percentile subgroups.

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Under-nutrition and Related Factors among Children Aged 6-59 Months in Gida Ayana District, Oromiya Region, West Ethiopia: a Community Based Quantitative Study

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Abstract

Background: Under-nutrition is the most known significant public health problems in developing countries of the world including Ethiopia that cause a shocking effect on children under five years of age. However, the extent of the problem and its several risk factors were not uniformly addressed enough across the various corner of the country. Thus, the objective of this study was to estimate the extent of under-nutritional status and to identify factors related to under-nutrition among children aged 6-59 months in Gida Ayana district, West Ethiopia.

Method: A community based cross sectional study was conducted in August/September, 2015. A total of 588 children aged 6-59 months with their respective mothers/caregivers/were used for this study. Weight, height and Mid-upper Arm Circumference (MUAC) were measured using instruments that are recommended by UNICEF (United Nations Children's Fund) and data regarding socioeconomic, demographic, child and maternal characteristics were collected using a pretested structured questionnaire through interview. Statistical Package for Social Sciences (SPSS) software version 20.0 was used to perform descriptive statistics as well as to perform bivariate and multivariate logistic regression analysis to identify factors related to under-nutritional status of children. World Health Organization (WHO) Anthro 2007 software version 1.0.4 was used to analyze anthropometric indices.

Results: The overall prevalence of stunting, wasting, and underweight were 40.5%, 10.9% and 19.2% respectively. In addition, prevalence of severe stunting; wasting and underweight were 13.4%, 2.9% and 4.6% respectively. Wasting was significantly higher in male children, 24-35 months aged children, House Holds (HHs) of illiterate fathers and HHs with lack of access to safe drinking water. Stunting were significantly higher in male children, children aged 36-59 months, HHs with lack of ownership of farm animals, children with diarrhea, children with fever and HHs with low monthly income. In addition, male children, urban children, children aged 24 months and above, HHs having more than one under five children and diarrhea were the factors that showed significant association with underweight.

Conclusion: The prevalence of wasting and stunting among under five children were high confirming nutritional situation in the study area is serious. Wasting, stunting and underweight were significantly higher among boys than girls. In addition; child age, residence, parent's formal education status, visiting antenatal clinic, and diarrhea becomes the main risk factors that contribute for the occurrence of at least two forms of under-nutritional status of children in the study area. Thus, efforts should be made to improve sources of drinking water and parental education, to prevent and control childhood illness, to implement child's age, residence and sex specific interventions as well as to establish therapeutic and supplementary feeding programs.

Keywords: Ethiopia; Gida Ayana; Under five children; Under-nutritional status; Underweight; Wasting; Stunting

Introduction

Under-nutrition is a pathological condition brought about by the inadequate intake of one or more of the essential nutrients necessary for survival, growth and reproduction [1]. It is the most known significant public health problems worldwide which becomes the underlying cause of 45% of deaths in children below 5 years of age [2,3]. In Sub-Saharan Africa, it is estimated that 4.8 million children die each year before reaching age of 5 years due to underling potentiating effect of under-nutrition on common infectious diseases, such as pneumonia and diarrhea [4]. Similarly in Ethiopia about 17% will die each year before reaching their fifth birthday due to underlying effect on common infectious diseases [5]. Other impacts of under-nutrition on children includes: retarding growth, diminish the immune system and enhancing susceptibility to infections, impaired mental development, and further enhancing the chance of under-nutrition [6]. It has also an impact on learning ability and productivity, thereby affecting the economic growth of the country.

There are various epidemiological studies done in different regions that showed the extent of the problem and associated risk factors. Reports from 2011 joint UNICEF-WHO-World Bank malnutrition estimation rate showed the prevalence of stunting and underweight in least developed countries (LDCs) was 38% and 23% respectively [7]. Contemporaneously other study found the prevalence of stunting and underweight among under five children in sub-Saharan Africa were 38% and 28% respectively [8]. In Ethiopia, according to Ethiopia Mini

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Demographic and Health Survey (EMDHS), 2014 40% of under five children were stunted, 10% wasted and 25% underweight. Similarly in Oromia regional state, 38.2% of under five children are stunted, and 18.6% severely stunted, 22.7% of them underweight with 7% severe underweight and 7.1% of the under five children are wasted, and 1.7% severely wasted [9]. A study done in Kombolcha District of Eastern Hararghe, Ethiopia found that 45.8%, 28.9% and 11.2% of under five children were stunted, underweight and wasted respectively [10]. A study conducted in Dollo Ado district, Somali region, Ethiopia among children aged 6-59 months found that 42.3%, 34.4% and 47.7% of the children were wasted, stunted and underweight respectively [11]. A study conducted in Gumbrit, North West Ethiopia among preschool children found that 28.5%, 24% and 17.7% of the children were underweight, stunted and wasted respectively [12]. A cross sectional study conducted in Mecha and Wenberma Woreda of West Gojjam, Northern Ethiopia found that 43.2%, 14.8% and 49.2 of the children under age five were suffering from stunting, wasting and underweight respectively [13]. Up to the researchers knowledge there is no previous study conducted on under-nutrition and related factors among under five children in the selected study area that reveal the magnitude of the problem and factors leading to the problem. Thus, the objective of this study was to determine the prevalence of under-nutrition and related factors among children aged 6-59 months in Gida Ayana district, Oromiya region, West Ethiopia from August 11 to September 11, 2015. Hence, the results of this study will help to know the magnitude and related contributing factors of the problem. This will provide baseline data about nutritional status and health condition of the study target groups, where other related issue will relay on. Moreover, it could add knowledge to the existing evidences about these problems. Lastly, knowing the extent of the problem and identifying the risk factors related with nutritional status of under five children in the study area will enable to guide public health planners and policy makers in determining priorities, in designing appropriate and effective nutritional intervention programs to address the problem and its associated consequences.

Methods and Materials

Study design, study area and study period

A community based cross sectional study was conducted in Gida Ayana district, East Wollega zone, west Ethiopia from August 11 to September 11, 2015. Gida Ayana district has 28 Administrative Kebeles (AKs); 21 rural and 7 of the Kebeles are urban. The total population of the district is 131,982 of which 66,291 were male and 65,691 were female as of 2007 census [14]. The total number of under five children in the district can be estimated from the total population. In Ethiopia the estimated proportion of under five population is nearly 15.4% [14] and the estimated number of under five children becomes approximately 20,325. In addition the total number of households in the district was 27,496. The district has three climatic zones; low land, midland and highland. In the district, there is one district hospital with ambulance service, four health centers and 22 health posts. Each health post had at least one health extension workers who provide basic primary health care services. Except for few, the livelihood of the people residing in the district depends directly or indirectly on agriculture. Most of the farmers in the area cultivate crop mainly maize, sorghum, sesame, bolekie, gobe, groundnut and akuri ater.

Study variables

Dependent variables: Indicators of under-nutritional status which includes: Stunting, wasting and underweight among children aged 6-59 months.

Independent variables: The following categories of factors were studied

- Demographic and socio-economic variables; marital status of the mother, family size (total household members), total number of children under five years old, maternal/paternal education and occupation, income, number of livestock owned, farm land ownership and size.
- Child characteristics; age of child, child sex, height, weight, birth order, birth weight, birth interval and manifestations of childhood morbidity (diarrhea, fever and cough).
- Child caring practices; IYCF (infant and young child feeding) practices, hygiene, health care seeking behavior, immunization.
- Maternal characteristics and caring practices; age of mother, nutrition awareness, number of children ever born, ANC (antenatal clinic) visits, health status during pregnancy, use of extra food during pregnancy and autonomy in decision-making.
- Environmental Health condition; water supply, sanitation and housing conditions.

Operational definitions:

- ***Under-nutrition*** in this study refers to state resulting from a relative or absolute deficiency of one or more essential nutrients and manifested by stunting, wasting and underweight.
- ***Stunting*** refers height for age less than the international median WHO reference value by more than two standard deviations
- ***Sever stunting*** refers height for age below-3SD (Standard Deviation) of the median WHO reference values.
- ***Wasting refers*** weight for height less than the international median WHO reference value by more than two SD.
- ***Sever wasting*** refers weight for height below-3SD of the median WHO reference values.
- ***Underweight*** refers weight for age less than two SD below the international median WHO reference value.
- ***Severe underweight*** refers weight for age below-3SD of the median WHO reference values.
- ***Access to health facility*** refers to the availability of health care facilities for the clients within 10 km radius.
- ***Household*** is defined as those people living together under 1 roof and sharing a common kitchen.
- ***IYCF practices*** which includes pre-lacteal feeding, time of initiation of breast feeding, feeding of colostrum, duration of exclusive breast feeding, duration of breast feeding, age at complementary feeding, the type and frequency of complementary foods and methods of complementary feeding.
- ***Pre-lacteals*** is defined as any feeding given to babies before initiating breast-feeding for the first time after birth.
- ***Exclusive breast feeding*** refers feeding only breast milk without anything else for the first six months of life, with the exception of medicines for therapeutic purpose.

- ***Complementary foods*** are foods which are required by the child, at and above six months of age, in addition to sustained breastfeeding.
- ***Low birth weight*** has been defined by as weight at birth of <2500 grams.
- ***Diarrhea*** refers passage of loose stools for three or more times in a day.
- ***Fever*** refers elevated body temperature than usual.

Source population: The source population was all children aged 6-59 months and their mothers/care givers/living in Gida Ayana district during the study.

Study population: The study population was all children aged 6-59 months and their mothers/care givers/in the selected HHs who fulfill inclusion criteria.

Inclusion criteria

- Children aged 6-59 months and their mother/care giver/living in the area for at least 6 months prior to the study was included in the study.

Exclusion criteria

- Children and their mothers/care givers/who had serious illness and/or hospitalized for diseases was not included in the study.
- Children and their mothers/care givers/having mental illness and physical deformity like deformity of upper limb, deformity of lower limb, deformity of thoracic region both anteriorly and posteriorly and making difficult for measurement was excluded from the study.

Sample size determination

The sample size was calculated using single population proportion determination formula; $n=Z^2\ (\alpha/2)\times p\times(1-p)/d^2$, by taking the prevalence of stunting, underweight and wasting among under five children respectively 41.78%, 39.6% and 11.84% from previous local study [15] and based on the assumption of 95% confidence interval and a margin of error of 5%. After considering design effect of 1.5 and adding 5% non response rate the final sample size respectively is becoming 588.7035~588, 578.8755~578 and 252.6237~252.

Sampling procedures

The sampling technique used to select appropriate and representative sample was two-stage by using simple random sampling method (lottery and Microsoft office excel generated random number). First the representative AKs in the district was selected by lottery. In the selected AKs of the district households having under five children were identified with the help of health extension workers (HEWs) and community leaders. The names of identified HHs having under five children were coded by number. Then this HHs was selected by random number (Microsoft office excel generated) in each study AKs of the district proportional to the estimated HH size having under five children and mother-child pairs was taken for the interview and measurements. In those households having more than one under five children, one child was selected by lottery method (Figure 1).

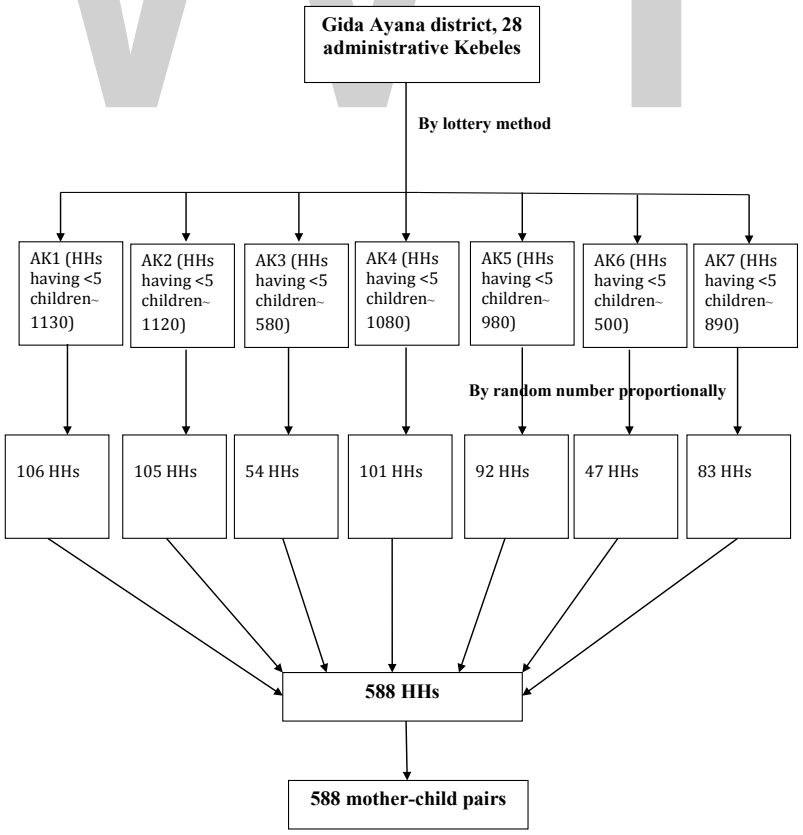

Figure 1: Schematic presentation of sampling procedures.

Data Collection Instruments and Procedures

The data was collected using pretested structured questionnaire and anthropometric measurements. The questionnaire was initially prepared in English and then translated into the local language, Afan Oromo, by fluent speakers of both languages and again it was translated back into English to check its consistency. Information regarding to socioeconomic and demographic factors, child factors, maternal factors and environmental health conditions was collected from mothers/caregivers by face to face interview using pretested structured questionnaire through house-to-house visit.

Anthropometric Measurements

Anthropometric data was obtained by measuring weight and height of children. Weight was measured without any footwear and with minimal clothing to the nearest 0.1 kg using recommended UNICEF weighing scale. In the time of refuse to be scaled, children's mothers were carrying and stand on the scale. Then, the child actual weight was obtained by subtracting mother's weight from mother and child weight. Standing height for those who is 24 months and older was measured without any footwear to the nearest 0.1 cm using a standard calibrated bar. The children were made to stand straight with heels, buttocks, shoulders and back of head touching the wall. Head hold comfortably erect with the lower border of orbit of the eye in the same horizontal plane as the external canal of the ear and the arms hanging loosely by the sides with palms facing the thigh. Measurement was read by placing the horizontally hold wooden board/scale touching the top of the head. The height was compared with the new WHO child growth standards, 2006 reference data for that particular age and sex to get height for age. Children below 24 months of age (below 85 cm) was measured in a recumbent position by using a length board with a headpiece to the nearest 0.1 cm. Heads touch the headpiece with their back, back of knees and heels touching the board and their hands be relaxed during measuring.

In addition the correct age of a child was elicited from the child's vaccination card or discharge delivery card and mother's recall. Mother's recall especially recall of illiterate mother was assisted by referring to local events like traditional festivals/ceremonies that took place around the period they gave birth to their children. In addition to strength the quality of data measurement MUAC was performed. Mid upper arm circumference (MUAC) was measured on left mid upper arm using flexible measuring tape to the nearest 0.1 cm.

Data Quality Management

To maintain data quality educated individuals completing 1st degree and graduating university students especially who do have previous experience of data collection and who speak local languages spoken in the study area was selected for data collection and supervision. Prior to the actual data collection, three day intensive training was given to interviewers and supervisors focusing on the rationale and objectives of the study, administration of the structured questionnaire, survey instruments, anthropometric measurements and ethical considerations.

Moreover, pre-test of questionnaires was done before the actual data collection work, by using 5% of the sample size on those people who was not included in the study to see for the accuracy of responses, to estimate time needed and some modifications was made on the basis of the findings. Weighing scales was calibrated with known weight object regularly. The scales indicators were checked against zero reading after weighing every child. On daily basis collected information was checked for completeness and consistency by the supervisors and by principal investigator to keep the quality of data and possible errors was returned to the data collectors for correction. Data validity and reliability was maintained through close supervision of the measurements by the principal investigator and trained supervisor. To minimize sampling error, weight and height of the children was taken two times by the same person and the average value was taken for final analysis.

Data Processing and Analysis

Data was coded and entered in to Epi Data version 3.1 statistical package software by one trained data clerk and by the principal investigator and it was exported to Statistical Package for Social Sciences (SPSS) software version 20.0 for analysis of descriptive statistics and statistical inferences. Data cleaning and editing was made before analysis.

Characteristics of the sample like socioeconomic and demographic factors, child factors, maternal factors and environmental health conditions was described in terms of frequencies and percentages through texts, tables and graph. Both bivariate and multivariate logistic regression analysis was performed to identify the factors that are associated with child under nutritional status. All the variables with p value ≤ 0.2 at the bivariate analysis were entered into the multivariable logistic regression model. In addition, repeatedly reported risk factors of poor nutritional status like perceived size of baby at birth, presence of diarrhea within 2 weeks of the survey, and pre-lacteal feeding practices were entered into the model regardless of the p-value. In multivariable logistic regression analysis OR (odds ratio) and 95% CI (confidence interval) was estimated to reveal the strength of association and a "p" value less than 0.05 was used to declare the statistical significance. Anthropometric indices H/A (Height-for-age), W/H (Weight-for-Height) and W/A (Weight-for-Age) taking age and sex into consideration was calculated using WHO Anthro 2007 software version 1.0.4.

Results

Demographic and socio-economic characteristics of the studied children, their mother and their households

All of the planned study subjects were participated in the study, making the response rate 100%. About 60% of respondents are living in rural area. Among the children studied, 293 (49.8%) were male and 295 (50.2%) were female, 145 (24.7%) fell in the age group 12-23 months. The median age of the children studied was 24 months. About 102 (17.3%) of the studied children were born to mothers aged less than 20 years. Mothers who gave first birth at their age 18 or less years were 56.5%. Average total number of children born to a mother was 2.9 with 2.2 SD, and 21.8% of the mothers gave birth of five and above children. Out of the total interviewed mothers 550 (93.5%) were married, 377 (64.1%) were illiterate and 512 (87.1%) were housewife. On the other hand 336 (59.1%) of mothers husband were literate and 354 (65%) of them were farmer in occupation. Almost half of (51.5%) of the study participants were Muslim and 61.7% were Oromo ethnic group.

About 41 HHs are headed by female and average household size is 4.8 persons with 1.73 SD and 31% of the HHs has more than five HH size. Sixteen % of the HHs had two under five year children and about 2 HHs had three under five year children. The detailed demographic and socioeconomic characteristics of the sample children, their mothers and their HHs are presented in Tables 1 and 2.

Health and health related characteristics of the studied children, their mothers and their households

The common childhood illnesses identified in the study were diarrhea, acute respiratory infections, malaria and typhoid fever. In about 211 (52.8%), 196 (49%) and 46 (11.5%) under five children respectively, complaints of diarrhea, complaints of fever and complaints of respiratory diseases were reported within 2 weeks preceding the study.

The mean amount of water used in a day per household was 60.2 liters with SD of 16.5 and almost all (95.7%) of HHs use more than 20 liters per day. Almost all (98%) of HHs have latrine and the commonest type (70.3%) utilized were traditional private pit latrine with wooden slab. The other detailed health and health related characteristics of the sample children, their mothers and their households are presented in Table 3.

Feeding practices of the studied children and their mothers

About 416 (70.7%) mothers started breastfeeding immediately after birth. Out of 342 (82.2%) mothers who have information about immediate breast feeding majority (63.4%) got from health extension workers. About 197 (33.5%) of respondents give pre-lacteal food/fluid for their child and the commonest ingredients given were water, cow milk and butter. Almost all of currently breast feed mothers provide the breast milk in both day and night time for more than 8 times per day. The other feeding practices of the sample children and their mothers are presented in Table 4.

Nutritional status of the studied children

Overall prevalence of stunting, wasting, underweight and overweight were 40.5%, 10.9%, 19.2% and 1.2% respectively and there were no cases of obesity. In addition prevalence of severe stunting; wasting and underweight were 13.4%, 2.9% and 4.6% respectively. Moreover, as measured by MUAC 13 (2.2%) studied children were severely wasted (in severe acute under nutritional status) (MUAC<11.5 cm) and 71 (12.1%) were moderately wasted (in moderate acute under nutritional status) (MUAC<12.5 cm) (Figure 2) (Tables 5 and 6).

Analysis result of factors associated to child nutritional status

The binary logistic regression analysis revealed selected demographic, socio-economic, health and health related factors as well as selected child feeding practices are associated to under nutritional status of children (stunting, wasting and underweight) and presented in Tables 7- 9 below. Sex, age and paternal education status were among the variables which showed a significant association with wasting, stunting and underweight ($p<0.05$).

Factors associated with wasting

This study revealed child sex, child age, residence, paternal education status, number of antenatal clinic visits and sources of drinking water were significantly associated with wasting.

The occurrence of wasting were 2.1 times higher in male children [AOR (adjusted odds ratio)=2.1, 95% CI=1.03, 4.12] as compared to female counterparts. Children in age group 24-35 months were 4.3 times at risk of being wasted (AOR=4.3, 95% CI=1.38, 13.39) than those older children. Children living in urban area were 1.96 times at risk of being wasted [COR (crude odds ratio)=1.96, 95% CI=1.16, 3.3] than those living in rural area. The risk of being wasted were 2.4 times higher in those children of illiterate fathers (AOR=2.4, 95% CI=1.22,

Variables	Characteristics	Frequency	Percent
Child's sex (n=588)	Male	293	49.8
	Female	295	50.2
Child's age (n=588)	6-11	97	16.5
	12-23	145	24.7
	24-35	109	18.5
	36-47	121	20.6
	48-59	116	19.7
Birth order (n=588)	1	175	29.8
	2-4	296	50.3
	>4	117	19.9
Birth interval (n=413)	<2 years	126	30.5
	Every 2 years	70	16.9
	>2 years	217	52.5
	Larger	86	14.6
Perceived size of baby at birth (n=588)	Average	308	52.4
	Small	194	33
Total number of children born to a mother (n=588)	<5 children born in a mother	460	78.2
	≥5 children born in a mother	128	21.8
Mothers age (n=588)	≤18 years	21	3.6
	>18 years	567	96.4
Mothers age at first birth (n=588)	≤18 years	332	56.5
	>18 years	256	43.5
Mother age during birth of index child (n=588)	<20years	102	17.3
	20-29years	351	59.7
	30-39years	130	22.1
	≥40years	5	0.9
Current marital status (n=588)	Single	13	2.2
	Married	550	93.5
	Divorced	21	3.5
	Widowed	4	0.7
Maternal formal education (n=588)	Yes	211	35.9
	No	377	64.1
Maternal formal education level (n=211)	Primary 1st cycle (1-4 grade)	93	44.1
	Primary 2nd cycle (5-8 grade)	80	37.9
	High school and above	38	18
Paternal formal education (n=569)	Yes	336	59.1
	No	233	40.9
Paternal formal education level (n=336)	Primary 1st cycle (1-4 grade)	136	40.5
	Primary 2nd cycle (5-8 grade)	129	38.4
	High school and above	71	21.1
Mother occupation (n=588)	Housewife only	512	87.10%
	Farmer	242	41.20%
	Merchant/trade	59	10.00%
	Private organization employee	3	0.50%
	Government employee	5	0.90%
	Daily laborer	19	3.20%
	Farmer	354	62.20%
	Merchant/trade	132	23.20%
Husband occupation (n=569)	Private organization employee	54	9.50%
	Daily laborer	27	4.80%
	Government employee	16	2.80%

Ethnicity (n=588)	Oromo	363	61.7
	Amhara	181	30.8
	Tigre	43	7.3
	Orthodox	220	37.4
Religion (n=588)	Muslim	303	51.5
	Protestant	65	11.1

Table 1: Demographic and Socio-economic characteristics of the sample children and their mothers in Gida Ayana district, East Wollega Ethiopia, August/September 2015.

Variables	Characteristics	Frequency	Percent
Residence (n=588)	Urban	235	40
	Rural	353	60
Household head (n=588)	Mother	41	7
	Father	547	93
HH size (n=588)	2-5	406	69
	>5	182	31
<5 years children in a HH (n=588)	1	492	83.7
	2	94	16
	3	2	0.3
Material of roof of the house (n=588)	Thatched	14	2.4
	Corrugated iron sheet	574	97.6
Material of floor of the house (n=588)	Earthen/soil	513	87.3
	Cemented	75	12.8
Presence of windows (n=588)	Yes	570	96.9
	No	18	3.1
Monthly HH income (in ETB, n=588)	≤1500	166	28.2
	1501-3001	264	44.9
	>3001	158	26.9
Decision making on utilization of money (n=588)	Mainly husband	245	41.7
	Both jointly	195	33.2
	Only husband	87	14.8
	Mainly wife	31	5.3
	Only wife	30	5.1
Ownership of farm animals/ livestock/ (n=588)	Yes	212	36.1
	No	376	63.9
Ownership of agricultural land (n=588)	Yes	360	61.2
	No	228	38.8
Ownership of agricultural land by hectare (n=360)	<5 hectare	261	72.5
	≥5 hectare	99	27.5
Currently cultivating crops on their farm land (n=360)	Yes	357	99.2
	No	3	0.8
Types of crops cultivated (n=357)	Maize	356	99.20%
	Akuri ater	331	92.20%
	Gobe	189	52.60%
	Selit	161	44.80%
	Groundnut	160	44.60%
	Bolekie	144	40.10%
	Coffee & khat	4	1.10%

Table 2: Demographic and socio-economic characteristics of the children's HHs in Gida Ayana district, East Wollega Ethiopia, August/September 2015.

Variables	Characteristics	Frequency	Percent
Ever faced any health problem of the child (n=588)	Yes	400	68
	No	188	32
Common childhood illnesses (n=400)	Malaria	100	25
	ARI (pneumonia)	140	35
	Diarrhea	290	72.5
	Typhoid	184	46
Diarrhea, preceding 2 wks (n=400)	Yes	211	52.8
	No	189	47.3
Frequency of diarrhea/ day (n=211)	1 episodes	12	5.69
	2 episodes	36	17.1
	3-4 episodes	123	58.3
	≥5 episodes	40	18.96
Fever, preceding 2 wks (n=400)	Yes	204	51
	No	196	49
Respiratory diseases, preceding 2wks (n=400)	Yes	46	11.5
	No	354	88.5
Ever taking to HF for sickness (n=400)	Yes	350	87.5
	No	50	12.5
Immunization status (n=588)	Immunized	557	94.7
	Not immunized	31	5.3
Source of drinking water (n=588)	protected sources[1]	384	65.3
	unprotected sources[2]	204	34.7
Time spent to fetch water (n=588)	<15 minutes	166	28.2
	15-30 minutes	355	60.4
	>30minutes	67	11.4
Presence of latrine (n=588)	Yes	576	98
	No	12	2
Methods of waste disposal (n=588)	open field disposal	270	45.9
	in a pit	44	7.5
	common pit	161	27.4
	Composting	15	2.6
	Burning	143	24.3
Perceived health status during pregnancy (n=588)	Good	450	76.5
	Not good/sick	138	23.5
Antenatal clinic visits (index child) (n=588)	None	113	19.2
	1-3 times visit	292	49.7
	≥4 times visit	183	31.1
Delivery place (index child) (n=588)	Home	386	65.6
	Health institution	202	34.4

1: Public tap, private pipe, protected spring; 2: River, pond, unprotected spring.

Table 3: Health and health related characteristics of the sample children, their mothers and their households in Gida Ayana district, East Wollega Ethiopia, August/September 2015.

Variables	Characteristics	Frequency	Percent
Child receive pre-lacteal foods/fluids (n=588)	Yes	197	33.5
	No	391	66.5
Commonest pre-lacteal foods used (n=197)	Water	16	8.1
	Butter	18	9.1
	Cow milk	162	82.2
	Other	1	0.5
Child feed 1st milk (n=588)	Yes	145	24.7
	No	443	75.3
Currently breastfeeding (n=588)	Yes	285	48.5
	No	303	51.5
Given additional foods preceding 24 h of the survey (n=285)	Yes	250	87.7
	No	35	12.3
Age initiated for CF (n=250)	<4 month	3	1.2
	4-6 month	201	80.4
	7-12 month	46	18.4
Types of CF initiated (n=250)	Cow's milk	156	62.4
	Butter	13	5.2
	Sugar solution	61	24.4
	Formula milk	32	12.8
	Attmit	188	75.2
	Injera and bread	145	58
Frequency of CF/day (n=250)	<3 times	10	4
	3 times	138	55.2
	>3 times	102	40.8
Methods of CF (n=250)	Bottle	113	45.2
	Cup	167	66.8
	Spoon	120	48
	Hand	121	48.4
Duration of BF (n=303)	<12 months	9	3
	12-24 months	219	72.3
	>24 months	75	24.8
Who cares, baby feeding (n=588)	Mother	548	93.2
	Grandmother	10	1.7
	Others	30	5.1
Change in feeding practice during illness (n=588)	Yes	373	63.4
	No	215	36.6
Feeding practices during illness (n=373)	preventing breast	16	4.3
	preventing food	157	42.1
	Providing additional food	200	53.6
Hand wash during preparation and feeding of child and herself (n=588)	Wash using water only	142	24.1
	Wash using soap some times	223	37.9
	Wash using soap always	189	32.1
	No wash	34	5.8
Extra food consumption during pregnancy/ lactation (n=588)	Yes	384	65.3
	No	204	34.7

Table 4: Feeding practices of the sample children and their mothers in Gida Ayana district, East Wollega Ethiopia, August/September 2015.

Nutritional status	Prevalence	95% CI
Stunting	40.5	(36.6, 44.6)
Wasting	10.9	(8.3, 13.8)
Underweight	19.2	(16.0, 22.8)
Overweight	1.2	(.3, 2.0)
Obesity	0	0

Table 5: Nutritional status of the studied children as measured by stunting, wasting, underweight , overweight and obesity**,** Gida Ayana district, East Wollega Ethiopia, August/September 2015 (n=588).

Nutritional status	Prevalence	95% CI
Severe acute under nutrition (MUAC<11.5)	2.2	(1.0, 3.4)
Moderate acute under nutrition (MUAC<12.5)	12.1	(9.5, 14.8)
Normal (MUAC ≥ 12.5)	85.7	(82.8, 88.4)

Table 6: Nutritional status of the studied children as measured by mid upper arm circumference (MUAC)**,** Gida Ayana district, East Wollega Ethiopia, August/ September 2015 (n=588).

Variables and characteristics	Wasting No (%)	COR (95% CI)	AOR (95% CI)
Residence			
Urban (n=235)	35 (14.9%)	1.955 (1.159, 3.298)*	1.407 (0.706, 2.804)
Rural (n=353)	29 (8.2%)	1	1
Child sex			
Male (n=293)	47 (16.0%)	3.124 (1.748, 5.584)**	2.059 (1.028, 4.122)*
Female (n=295)	17 (5.8%)	1	1
Child age			
6-11 (n=97)	12 (12.4%)	1.678 (0.676, 4.170)	2.894 (0.866, 9.669)
12-23 (n=145)	13 (9.0%)	1.171 (0.482, 2.844)	2.177 (0.681, 6.959)
24-35 (n=109)	23 (21.1%)	3.180 (1.399, 7.228)*	4.296 (1.378, 13.392)*
36-47 (n=121)	7 (5.8%)	0.730 (0.263, 2.029)	0.614 (0.128, 2.943)
48-59 (n=116)	9 (7.8%)	1	1
Paternal formal education			
Yes (n=336)	28 (8.3%)	1	1
No (n=233)	33 (14.2%)	1.815 (1.064, 3.096)*	2.404 (1.224, 4.722)*
Maternal education level			
Primary education (n=173)	22 (12.7%)	1.7 (0.482, 5.999)	1.137 (0.516, 2.504)
High school and above (n=38)	3 (7.9%)	1	1
Perceived size of baby at birth			
Large (n=86)	8 (9.3%)	1	1
Average (n=308)	42 (13.6%)	1.539 (0.694, 3.416)	2.824 (0.857, 9.303)
Small (n=194)	14 (7.2%)	0.758 (0.306, 1.881)	1.159 (0.306, 4.397)
Diarrhea, preceding 2wks			
Yes (n=211)	20 (9.5%)	1	1
No (n=189)	30 (15.9%)	1.802 (0.985, 3.295)	2.004 (0.996, 4.032)
No. of antenatal clinic (ANC) visit			
1-3 times (n=183)	28 (15.3%)	1	1
≥4 times (n=292)	27 (9.2%)	0.564 (0.321, 0.992)*	0.707 (0.329, 1.516)
Source of drinking water			
Unprotected (n=204)	34 (16.7%)	2.360 (1.398, 3.985)**	2.991 (1.485, 6.025)**
Protected (n=384)	30 (7.8%)	1	1
*p<0.05; **p<0.005; COR: Crude odds ratio; AOR: Adjusted odds ratio; CI: Confidence interval			

Table 7: Bivariate and multivariate binary logistic regression analyses results which show the effect of selected variables on nutritional status as measured by wasting, Gida Ayana district, East Wollega Ethiopia, August/ September 2015.

Variables and characteristics	Stunting No (%)	COR (95% CI)	AOR (95% CI)
Child sex			
Male (n=293)	124 (42.3%)	1.17(0.84, 1.62)	1.602 (1.014, 2.529)*
Female (n=295)	114 (38.6%)	1	1
Child age			
6-11 (n=97)	28 (28.9%)	1	1
12-23 (n=145)	60 (41.4%)	1.74 (1.004, 3.01)*	1.431 (0.690, 2.967)
24-35 (n=109)	42 (38.5%)	1.545 (0.861, 2.772)	1.330 (0.614, 2.884)
36-47 (n=121)	55 (45.5%)	2.054 (1.165, 3.619)*	3.055 (1.403, 6.650)**
48-59 (n=116)	53 (45.7%)	2.073 (1.171, 3.670)*	2.376 (1.083, 5.213)*
Maternal formal education			
Yes (211)	68 (32.2%)	1	1
No (377)	170 (45.1%)	1.73 (1.21, 2.46)**	1.533 (0.911, 2.579)
Ownership of animals			
Yes (n=212)	73 (34.4%)	1	1
No (n=376)	165 (43.9%)	1.49 (1.05, 2.11)*	1.765 (1.071, 2.909)*
Paternal formal education			
Yes (n=336)	122 (36.3%)	1	-
No (n=233)	106 (45.5%)	1.46 (1.04, 2.06)*	1.086 (0.676, 1.743)
Monthly HH income			
≤1500 (n=166)	83 (50.0%)	1.508 (.971, 2.342)	2.715 (1.397, 5.276)**
1501-3001 (n=264)	92 (34.8%)	0.807 (0.537, 1.211)	0.932 (0.537, 1.618)
>3001 (n=158)	63 (39.9%)	1	1
Child receive pre-lacteal foods/fluids			
Yes (n=197)	84 (42.6%)	1.144 (0.808, 1.620)	1.005 (0.617, 1.638)
No (n=391)	154 (39.4%)	1	1
Diarrhea, preceding 2wks			
Yes (n=211)	101 (47.9%)	1.597 (1.07, 2.384)*	2.377 (1.431, 3.946)**
No (n=189)	69 (36.5%)	1	1
Child had fever in last two weeks			
Yes (n=204)	93 (45.6%)	1.295 (0.870, 1.927)	1.754 (1.057, 2.909)*
No (n=196)	77 (39.3%)	1	1
Perceived size of baby at birth			
Large (n=86)	37 (43.0%)	1	1
Average (n=308)	112 (36.4%)	0.757 (0.466, 1.230)	0.570 (0.280, 1.161)
Small (n=194)	89 (45.9%)	1.123 (0.673, 1.873)	1.192 (0.563, 2.523)
Antenatal clinic visits			
Yes (n=475)	179 (37.7%)	1	1
No (n=113)	59 (52.2%)	1.807(1.195, 2.731)**	1.596 (0.909, 2.803)
*p<0.05; **p<0.005			

Variables and characteristics	Under-weight No (%)	COR (95% CI)	AOR (95% CI)
Residence			
Urban (n=235)	51 (21.7%)	1.301 (0.860, 1.968)	2.204 (1.081, 4.495)*
Rural (n=353)	62 (17.6%)	1	1
Child sex			
Male (n=293)	63 (21.5%)	1.342 (0.888, 2.028)	2.042 (1.138, 3.662)*
Female (n=295)	50 (16.9%)	1	1
Child age			
6-11 (n=97)	9 (9.3%)	1	1
12-23 (n=145)	19 (13.1%)	1.474 (0.637, 3.410)	2.012 (0.708, 5.716)
24-35 (n=109)	30 (27.5%)	3.713 (1.661, 8.301)**	3.601 (1.284, 10.098)*
36-47 (n=121)	26 (21.5%)	2.676 (1.189, 6.025)*	3.133 (1.068, 9.189)*
48-59 (n=116)	29 (25.0%)	3.259 (1.458, 7.286)**	3.968 (1.396, 11.275)*
Maternal formal education			
Yes (n=211)	31 (14.7%)	1	1
No (n=377)	82 (21.8%)	1.614 (1.026, 2.539)*	1.872 (0.921, 3.804)
Ownership of farm land			
Don't have (n=228)	38 (16.7%)	0.760 (0.494, 1.170)	0.614 (0.287, 1.310)
Have (n=360)	75 (20.8%)	1	1
Paternal formal education			
Yes (n=336)	53 (15.8%)	1	1
No (n=233)	57 (24.5%)	1.729 (1.138, 2.628)*	1.742 (0.985, 3.079)
Family size			
2-5 (n=406)	71 (17.5%)	1	1
≥6 (n=182)	42 (23.1%)	1.415 (0.921, 2.175)	1.457 (0.708, 3.000)
No of <5 children/HH			
1 (n=492)	89 (18.1%)	1	1
≥2 (n=96)	24 (25.0%)	1.509 (0.901, 2.528)	2.257 (1.126, 4.524)*
Birth order			
1 (n=175)	26 (14.9%)	1	1
2-4 (n=296)	69 (23.3%)	1.742 (1.061, 2.861)*	2.009 (0.941, 4.287)
>4 (n=117)	18 (15.4%)	1.042 (0.543, 2.001)	0.672 (0.225, 2.003)
Child receive pre-lacteal foods/fluids			
Yes (n=197)	45 (22.8%)	1.406 (0.921, 2.147)	1.623 (0.899, 2.931)
No (n=391)	68 (17.4%)	1	1
Diarrhea, preceding 2wks			
Yes (n=211)	52 (24.6%)	1.881 (1.130, 3.128)*	2.228 (1.166, 4.256)*
No (n=189)	28 (14.8%)	1	1
Child had fever in last two weeks			
Yes (n=204)	44 (22.4%)	1.351 (0.826, 2.210)	0.893 (0.481, 1.657)

Table 8: Bivariate and multivariate binary Logistic regression analyses results which show the effect of selected variables on nutritional status as measured by stunting, Gida Ayana district, East Wollega Ethiopia, August/September 2015.

No (n=196)	36 (17.6%)	1	1
Source of drinking water			
Protected (n=384)	82 (21.4%)	1.515 (0.963, 2.385)	1.812 (0.943, 3.483)
Unprotected (n=204)	31 (15.2%)	1	1
Immunization			
Immunized (n=557)	103 (18.5%)	1	1
Not immunized (n=31)	10 (32.3%)	2.099 (0.959, 4.592)	1.877 (0.661, 5.329)
*p<0.05; **p<0.005			

Table 9: Bivariate and multivariate binary Logistic regression analyses results which show the effect of selected variables on nutritional status as measured by underweight, Gida Ayana district, East Wollega Ethiopia, August/September 2015.

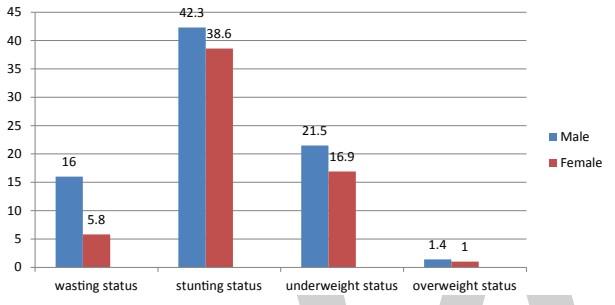

Figure 2: Percentage of wasting, stunting, underweight and overweight by child sex, Gida Ayana district, East Wollega Ethiopia, August/September 2015.

4.72) compared to those children of fathers attended formal education. Children of mothers who had greater number of antenatal clinic visit are 43.6% less likely to be wasted (COR=0.564, 95% CI=0.321, 0.992) as compared to those children of mothers who had less number of antenatal clinic visit, although this significance is marginal. Children who had no access to protected drinking water were 3 times at higher risk of being wasted (AOR=2.99, 95% CI=1.49, 6.03) than those who uses protected drinking water. Maternal education status, perceived size of baby at birth and diarrheal status within two weeks of the study were not significantly associated with wasting.

Factors associated with stunting

Analysis of this study showed child sex, child age, maternal and paternal education status, ownership of farm animals, monthly HHs income, diarrhea and fever preceding two weeks of data collection and antenatal clinic visits were significantly associated with stunting status.

The risk of being stunted were 1.6 times higher in male children (AOR=1.6, 95% CI=1.01, 2.53) than those female children. Children aged 12-23 months, 36-47 months and 48-59 months were 1.74 (COR=1.74, 95% CI=1.004, 3.01) times, 3.06 (AOR=3.06, 95% CI=1.40, 6.65) times, and 2.38 (AOR=2.38, 95% CI=1.08, 5.21) times at increased risk of being stunted respectively as compared to those aged 6-11 months. Children of illiterate mothers were 1.7 times at higher risk of being stunted (COR=1.73, 95% CI=1.21, 2.46) as compared to those children of mothers attended formal education. Similarly children of illiterate fathers were 1.5 times at higher risk of being stunted (COR=1.46, 95% CI=1.04, 2.06) as compared to those children of fathers attended formal education. Children of HHs which lack possession of farm animals were 1.8 times at increased risk of stunting (AOR=1.77, 95% CI=1.07, 2.91) compared to those children of HHs which possess farm animals. Children having complaint of diarrhea in the last two weeks preceding data collection were found to be 2.4 times at higher risk of stunting (AOR=2.377, 95% CI=1.431, 3.946). Similarly Children having complaint of fever in the last two weeks preceding data collection were found to be 1.75 times at higher risk of stunting (AOR=1.754, 95% CI=1.057, 2.909). Children of HHs having lower monthly income were 2.7 times at increased risk of stunting (AOR=2.72, 95% CI=1.397, 5.276). Children of mothers without antenatal clinic visit were 1.8 times at increased risk of stunting (COR=1.81, 95% CI=1.195, 2.731). Pre-lacteal feeding practice and perceived size of baby at birth were not significantly associated with stunting.

Factors associated with underweight

Analysis of this study showed child sex, child age, residence, maternal and paternal educational status, number of <5 children, birth order, diarrhea preceding 2 weeks of the study were found to be significant predictors of underweight.

The study showed, urban studied children were 2.2 times at higher risk of being underweight (AOR=2.20, 95% CI=1.08, 4.495) compared to those residing in rural area. Males are 2 times at increased risk of underweight (AOR=2.04, 95% CI=1.14, 3.66) compared to female counterparts. Children aged 24-35 months, 36-47 months and 48-59 months were 3.6 (AOR=3.6, 95% CI=1.28, 10.09) times, 3.1 (AOR=3.13, 95% CI=1.07, 9.19) times, and 4 (AOR=3.97, 95% CI=1.396, 11.28) times at increased risk of being underweight respectively as compared to those aged 6-11 months. Children with 2 up to 4 birth order were 1.7 times at increased risk of underweight (COR=1.742, 95% CI=1.061, 2.861) compared to those children born to mothers who had no previous birth. Children living in HHs having additional one or two under five children were 2.26 times at increased risk of underweight (AOR=2.257, 95% CI=1.126, 4.524) than those living in HHs without other under five children. Children having complaint of diarrhea in the last 2 weeks preceding data collection were found to be 2.2 times at higher risk of underweight (AOR=2.23, 95% CI=1.17, 4.26) compared to those children who didn't experience diarrhea 2 weeks preceding the study. In this study ownership of farm land, family size, pre-lacteal feeding practices, presence of fever within two weeks of the study, sources of drinking water and immunization status were not significantly associated with underweight.

Discussions

Stunting, wasting and underweight condition

Generally the prevalence of stunting, wasting and its severe forms are higher than 2014 EMDHS national as well as regional reports. But the figure of underweight and its severe form, sever stunting and overweight are lower than 2014 EMDHS national and regional reports. The high prevalence of wasting status may be attributed to unprotected drinking water sources that may lead to different infection and data collection period (August) when most of the households have shortage of food. Moreover, the prevalence of stunting, wasting, underweight and overweight were higher and lower than other studies conducted in different area of the world.

The prevalence of stunting, wasting and underweight is higher than compared to majority of the studies conducted abroad [16-22]. On the other hand, the prevalence of stunting, wasting and underweight is lower compared to reports of previous local studies conducted in eastern Hararghe, Somali region, west Gojjam, Guto Gida district [10,11,13,15]. Similarly the prevalence of wasting and underweight is lower compared to report of study done in Gumbrit, North west Ethiopia [12]. Even thought the extent of the problem is lower compared

to majority of previous local studies, stunting and wasting status in the study area was still serious condition during the study period.

Factors related to wasting, stunting and underweight

The result of this study showed, stunting was more prevalent in male children than female children which is consistent with previous many cross sectional studies conducted abroad and in Ethiopia [10,11,13,19,23-30]. This could be due to genetic deference and difference in energy requirement of boys and girls. In contrary, other studies reported that the occurrence of stunting among under five children was not significantly associated with child gender [12,21,31-33]. This study also showed, wasting and underweight were more prevalent in male children compared to female children which is consistent with other studies [23,34]. In contrary other studies report that wasting and underweight were more prevalent in female children than male children [10,22,35,36]. These discrepancies in findings could be attributed to differences in cultures, socioeconomic dynamics, parents' educational status and nutritional factors among the various communities. The discrepancies attributed to socioeconomic dynamics and educational status may be due to culture of the area in which the study is conducted may give priority for more care in any aspect for female children as well as the influence of sex preference of biological parents.

The finding of this study showed the prevalence as well as the risk of stunting and underweight increases with age. Children in age group 12-23 months and 36-59 months were at significantly higher risk of stunting compared to children in youngest age category. This finding is consistent with other studies [13,17,27]. In this study, significantly higher risk of underweight occurred in age group 24-35 months, 36-47 months and 48-59 months compared to youngest age group. This finding is consistent with previous studies [26,31,36]. The result of this study also showed the highest prevalence and significantly higher risk of wasting was occurred in age group 24-35 months compared to children in oldest age category. This finding is supported by previous study [15].

In this study maternal and paternal formal education status was significantly associated with stunting and underweight. Children of illiterate parents were at increased risk of stunting and underweight. This finding is consistent with the result of previous studies conducted elsewhere [19,22,26,37]. The result of this study also showed significant association between paternal formal education status and wasting. Children of illiterate fathers were at increased risk of wasting. This could be due to the fact that educated parents have the knowledge of improved child care, health services usage, hygiene and sanitation which have an impact on nutritional status of children. In contrary this study didn't find significant association between maternal education and wasting status. Contemporaneously, some of other previous studies didn't find significant association between maternal and paternal education and risk of stunting, wasting and underweight [12,18,33,38,39].

This study found children living in urban area were at significantly increased risk of wasting and underweight. The finding is consistent with previous studies conducted elsewhere [30]. This may be attributed to majority of HHs in urban area lack ownership of agricultural land and farm animals to cultivate different crops and lack of money to purchase the foods during time of data collection. This study also showed children of HHs which lacks ownership of farm animals are at significantly increased risk of stunting compared to those children of HHs which possess farm animals. This finding is consistent with previous study [11]. This probably attributed to HHs having farm animals can cultivate different crops and purchase/exchange/foods and other goods to be consumed by the children. On the other hand, this study found that farm land ownership had no significant association with any of the forms of under nutrition which is consistent with previous study [12].

Households' income level was significantly associated with chronic nutritional status. Children from households having low income are more likely to be stunted than those from households of high income. Previous studies conducted in different areas support this finding [11,18,19,24-26,30,40]. This could be due to high income households have greater purchasing power for food and other goods needed to ensure the health of children.

In this study children of mothers who hadn't antenatal clinic visit were at significantly increased risk of stunting compared to those children of mothers who had the visit. This finding is similar with those study conducted in Nepal [41] which showed children of mothers who hadn't antenatal clinic visit were at significantly increased risk of stunting compared to those who had the visits. These findings could be attributed to the health information given to mothers by health professionals during antenatal periods. Possible health information that health professionals could offer to mothers may include information on exclusive breastfeeding, initiation of complementary feeding as well as comprehensive care for the children.

Children with 2 up to 4 birth order were significantly at increased risk of underweight compared to those children born to mothers who had no previous birth. Another study conducted in Ethiopia [24] also showed children with birth order of 6 and above were at increased risk of underweight compared to those birth order of one. In contrast to this another study conducted in rural Bangalore [37] reported no significant association between underweight and birth order. This discrepancy may be due to difference in cultures, socioeconomic conditions, and parents' educational status of this various study area communities.

In this study, children living in HHs having additional one or two under five children were at significantly increased risk of underweight than those living in HHs without other under five children. This finding is supported by other study conducted in Butajira, Ethiopia [23] and trend analysis in Kenya [26] which reports HHs having other under five children was at increased risk of underweight. This probably attributed to food intake and accessibility of healthcare decrease with higher number of under five children especially in low income families.

Similar with the finding of other studies [13,18,24], in this study presence of diarrhea within two weeks preceding the data collection were significantly associated with the occurrence of stunting and underweight. The high prevalence and significant risk of stunting and underweight were observed among children who had experiencing diarrhea within two weeks preceding the study. Similarly consistent with other study [42], high prevalence and significant risk of stunting were observed among children who had experience fever within two weeks preceding the study. This may be attributed to vicious cycle relationship between infectious diseases and under nutritional status. Presence of diarrhea and fever causes loss of appetite and decreased intake of food which intern leads to weight loss and the children quickly become undernourished. On the other hand undernourished status further leads to the occurrence of diarrhea and other infectious diseases due to immune decline.

The finding of this study found that there is a significant association between sources of drinking water and acute nutritional status.

The higher prevalence and significant risk of wasting were occurred among children who utilized unprotected water sources. This finding is supported by previous studies [10,26,30]. The possible justification will be utilization of safe water decreases the probability of exposure of the child to water borne diseases that negatively affect the health and nutritional status of children in the study area.

In this study other commonly reported variables like pre-lacteal feeding practices as well as other IYCF practices (like deprivation of colostrum, EBF, duration of breast feeding, time of initiation of complementary feeding), family size and immunization status of children were not significantly associated with any of the indicators of under-nutritional status. This finding is consistent with other previous studies conducted elsewhere [18,33,38,39]. In contrary other previous studies reported the association of these factors with under-nutritional status indicators [10,11,13,24,30,37].

Conclusions

The result of this study found that indicators of under-nutritional status especially stunting and wasting were highly prevalent that confirms the nutritional condition in the study area is serious. The figure of wasting and stunting in the study area was higher than 2014 EMDHS national as well as regional figure.

This study found male children were at significantly increased risk of wasting, stunting and underweight. In addition the study revealed that among the risk factors; child age, residence, maternal and paternal formal education status, visiting antenatal clinic, and diarrhea becomes the main risk factors that contribute for the occurrence of at least two forms of under-nutritional status (wasting, stunting and underweight) of children in the study area. Thus, to tackle these problem efforts should be made to improve sources of drinking water and parental education, to prevent and control childhood illness, to implement child's age, residence and sex specific interventions as well as to establish therapeutic and supplementary feeding programs.

Declaration

Ethical consideration

Participants of the study were informed regarding the objectives of the study. Participation in the study was totally voluntary. Name and other personal identifiers were not recorded on data collection form and the information that they give us was kept confidential and was also used for this study purpose only. As the study was conducted through face to face interview and as well via measurement, it would not cause any harm as far as the confidentiality is kept. They were given full right to leave/to refuse to take part at any stage of the interview. But their participation in this study was essential to achieving the stated objectives that cannot be achieved without the participation of them. The response of the study participants enable to generate new knowledge that would produce benefits for themselves, for other persons or for society as a whole, or for the advancement of knowledge. Informed verbal consent was obtained from the participants as witnessed by data collectors name and signature. The proposal of this study was reviewed and approved by Ethical Review Committee of Wollega University. Permission was also obtained from the concerned bodies of East Wollega Zonal Health Department and the responsible administrative bodies of Gida Ayana district.

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Ghanaian Junior High School Adolescents Dietary Practices and Food Preferences: Implications for Public Health Concern

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Abstract

Introduction: The dietary practices of adolescents have been described as not the best, mainly as a result of their busy schedules, peer pressure and the independent nature of their behaviour. It is therefore important that adolescents have reliable nutrition information that will guide them to make informed decisions regarding their dietary patterns and practices. But, what are the gaps in their knowledge and practices regarding dietary intakes?

The aim of this study was to determine the eating patterns, meals skipping practices, snacking habits and the food preferences of adolescents in selected Junior High Schools in Ghana.

Methods: A total number of 820 adolescents were enrolled in this cross-sectional survey. A questionnaire assessing the background characteristics of the respondents, frequency of meals consumption, frequency of snacking between meals, type of snacks usually consumed, and frequency of eating outside the home and food preferences of respondents was administered to all the participants.

Results: Majority 515(62.8%) of the respondents indicated that they usually skipped breakfast before going to school. The common reason given by many 178(34.6%) of the breakfast skippers was that parents gave them money to buy food on their way to school, but they used the money to browse at the internet café after school. Nearly half 367(44.8%) of the respondents reported that they usually consumed an average of two cooked meals per day at home. About one-third (33.8%) of the respondents preferred a soft drink for snack during the day.

Conclusions: The findings of this study have demonstrated that Junior High School students, who are adolescents, do not have healthy eating patterns and habits – they usually skip breakfast and prefer high sugar and fat content food products as snack among other dietary habits.

Keywords: Adolescents; Dietary practices; Food preferences

Introduction

Adolescence has been described as the period of life between 11 and 21 years of age in which profound and dramatic biological, emotional and cognitive maturity is attained [1]. In this transitional stage of life, adolescents may no longer benefit from the attention and care usually given to children; and they may not get the protection associated with adulthood either. This transitional period between childhood and adulthood provides an opportunity to prepare for a healthy productive and reproductive life, and to prevent the onset of nutrition-related chronic diseases in adult life. It also affords an opportunity to adolescence-specific nutrition issues and, possibly, also corrects some nutritional problems originating in the past (World Health Organization [2]. There is therefore the need to know and understand the eating habits of adolescents, because of the high tendency for eating habits acquired during adolescence to persist into adulthood [3-6]. The adolescence period of life is therefore a critical period for establishing good dietary habits that would aid in the prevention of diseases in later life [4].

It has been indicated that adolescents are particularly vulnerable to nutrient inadequacies as their bodies undergo various physiological changes, and as they begin to become more socially independent, which often impacts negatively on their dietary intakes [7,8]. Some studies have also indicated that as a result of the rapid changes in physical growth and psychosocial development and as a result of the unhealthy dietary practices that adolescents adopt, they are unable to meet their dietary requirements. In addition, research has shown that, in most cases, healthy eating is not a priority of adolescents [9-11].

It is common knowledge that children and adolescents who develop healthy eating habits early in life are likely to maintain them into adulthood, and have a reduced risk of suffering from chronic diseases such as cardiovascular diseases, cancers, diabetes and osteoporosis [12]. Studies have also shown that adolescents who have healthy eating habits are more likely to have the ability to learn normally in school [13] and perform better academically than adolescents who have unhealthy eating habits [14,15].

Past studies have further revealed that adolescents frequently consume energy-dense diets which are of poor quality in terms of essential micronutrients [16-19]. The poor nutritional status of adolescents has been attributed to many factors, including low meal frequency, high consumption of sweetened beverages, increased consumption of energy-dense foods, increased consumption of foods away from home (with peers), skipping meals, particularly breakfast [16-18,20-24]. Other unhealthy practices include the consumption of high-dense fatty and sugary fast foods as the main meals of the day, eating meals characterized by a low content of fruits and vegetables, adopting unconventional dietary practices such as cutting down

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portion sizes of meals in an attempt to lose weight and attain a slim body figure, particularly among females [9-11,25].

Regarding snacking habits, it has been reported that adolescents usually cultivate the habit of consuming large portion sizes of fast food meals [26] and also consume high quantities of carbonated soft and energy drinks [27,28]. Although, ample evidence is not available, snacking has been linked to intakes of reduced portion sizes of meals, which makes it detrimental to health, since regular meal patterns are associated with healthier food choices and greater dietary diversity [29] and meeting recommended energy and nutrients intakes [30,31]. It has also been reported that adolescents who skip breakfast are most likely to have difficulty concentrating and remaining focused and alert in class by mid-morning. In addition, people who skip breakfast in particular are more likely to consume high sugar, fat and salt- dense snacks often during the day [32]. Similarly, other studies have shown that breakfast skipping is associated with substantially lower daily energy intakes [33,34]. In addition, other studies have found that children who practice unhealthy eating habits become more susceptible to obesity in early life, which later results in health defects such as cardiovascular diseases, diabetes and breast, colonic, endometrial and prostrate cancers [35,36].

The dietary practice of increasing intake of fast foods, replacing naturally nutritious high fibre diet with western diets which contain high concentrations of sugar and fat, coupled with the tendency to a more sedentary lifestyle, has resulted in the epidemic of childhood obesity [37-40]. Overweight and obesity, which were considered problems in high-income countries only, are dramatically rising in low and middle-income countries, particularly in urban settings, and hence have become global public health problems [35,41,42].

It has been asserted that the rising trend of overweight and obesity cases and their associated diseases among Ghanaians is likely to worsen, given the influx of high-energy dense foods into the Ghanaian market, coupled with the huge change in the dietary habits of people, mainly as a result of improvements in socio-economic conditions [43].

For many years, the health of adolescents has not been a major concern, and consequently, there has been limited research in the area of adolescent nutrition, particularly in developing countries such as Ghana. This is mainly due to the fact that adolescents are less susceptible to diseases and suffer from fewer life-threatening conditions than children and the elderly. Indeed, adolescence is generally described as a period of relatively good health, with low prevalence of infection and chronic diseases. In addition, mortality and morbidity trends among adolescents are quite similar in developing and developed countries. In addition, most health services in developing countries focus on children and pregnant women. As a consequence, in most cases, the health needs of adolescents may not be adequately investigated and addressed. However, the upsurge in the prevalence of childhood obesity worldwide has drawn much attention to the diets of adolescents and children [9]. Furthermore, it has been reported that adolescents are now at a high risk of becoming overweight and obese and liable to suffer from chronic diseases – particularly diabetes – owing to their unhealthy eating habits [44,45].

Research on basic dietary practices of adolescents in Ghana is scarce and can be described as almost non-existent. It is in this regard that this study was conducted to assess the dietary practices and food preferences of adolescents in some selected Junior High Schools (JHS) in Ghana. The findings of this study will serve as baseline information for the development of effective nutrition and health intervention programmes which will help address issues relating to unhealthy eating habits of adolescents not only in Ghana, but also in other developing countries battling with persistently upward trends in the incidence of non-communicable health conditions.

Methods

Study design and sample

This cross-sectional study was conducted in the Cape Coast Metropolis, in the Central Region of Ghana. In all, six JHS schools (three private and three public schools) were selected for the study. At the time of collecting the data all students at the school premises who consented to participate in the study completed the questionnaire administered.

Instrument

The items on the questionnaire were modified forms of items used in three similar studies [9,11,34]. The first section, (A), consisted of questions to assess the background characteristics of the respondents. Background information included sex, age, and type of school, living arrangement and household size, among other variables. In addition, participants were required to provide information regarding whether they were satisfied with their current size/ weight or not, and whether they had been taught any lesson on nutrition. The second section, (B), comprised 17 question items which assessed the dietary practices of the respondents. The items assessed frequency of meals consumption, frequency of snacking between meals, type of snacks usually consumed, and frequency of eating outside the home. The last section, (C), consisted of 12 food items in pairs; and the respondents were required to indicate their preferences from the list of pairs of food items given.

Data collection

The questionnaire was pretested prior to its administration in the study. The pretesting was among 60 adolescents in two other JHS schools not selected for the study; and it was modified for clarity on the basis of feedback obtained from the respondents. The questionnaire was administered by the researcher, with the help of trained research assistants and class teachers in all the six schools.

Ethical considerations

Permission was sought from head-teachers in all the selected schools prior to administration of the questionnaire. Consent forms were sent to parents through their children for permission for the children to participate in the study. Parental consent was obtained for 820 students, constituting 76% of all the eligible students. Prior to obtaining participants' consent, information sheets explaining the purpose of the study were distributed and explained to the students by the researchers.

Data analysis

Data collected was analyzed using the Statistical Package for Service Solution (SPSS) version 16.0. Descriptive statistics were run to summarize the data collected; and the results were displayed in frequencies and percentages for the variables being investigated. Chi-square tests were used to determine the presence of association between the variables. The chi-square test was run to assess for any statistical significant difference between males and females with respect to their dietary habits. The variables sex and all the different dietary practices variables were all categorical variables. A chi – square test which is a test for independence, evaluates statistically significant differences between proportions for two or more groups in a data set. A significance level

of ($p > 0.05$) was used. If the *P*-value was less than the significance level (0.05), the null hypothesis was rejected and a conclusion that there is a statistical difference between sex and the other dietary practices variables was made.

Results

Background characteristics of the respondents

The background characteristics of the respondents are presented in Table 1. The respondents comprised 449(54.8%) and 371(45.2%) students from private schools and public schools respectively. Of the 820 adolescent participants, with ages ranging from 11 to 17 years, a greater proportion 485(59.1%) were males. The majority 590(72.0%) of the respondents indicated that they often ate alone. Another 176(21.5%) indicated that they often ate at table with their family members; and 54(6.6%) reported that they often ate with their peers or friends. As to whether participants were satisfied with their body size or not, a large majority (80.0%) indicated that they were satisfied with their current body weight and size. Most (97.1%) of the respondents indicated that they had been taught topics on nutrition in school.

A high proportion (65.5%) of the students indicated that they had not heard about the Regenerative Health and Nutrition (RHN) Programme. The RHN is a preventive and promotive health-care programme initiated by the Ministry of Health (MOH) which aims to improve the health status of Ghanaians by emphasizing lifestyle changes, including what people should eat and drink, the need to increase one's physical activity levels, the importance of daily resting/sleeping and cleanliness.

Variables	n (%)
Type of School	
Private	449(54.8)
Public	371(45.2)
Form	
JHS1	219(26.7)
JHS2	265(32.3)
JHS3	336(41.0)
Sex	
Males	485(59.1)
Females	335(40.9)
Age Group	
13-11	396(48.3)
14-16	348(42.4)
>16	76(9.3)
Eating companions	
With family members	176(21.5)
With peers or friends	54(6.6)
Eats alone often	590(72.0)
Satisfied with body weight/Size and Shape	
No, wants to be bigger	84(10.2)
Yes, satisfied	656(80.0)
No, wants to be smaller	80(9.8)
Have been taught Nutrition in School	
Yes	796(97.1)
No	24(2.9)
Have heard about RHN* programme	
Yes	283(34.5)
No	537(65.5)

*RHN (Regenerative Health and Nutrition Programme)

Table 1: Background Characteristics of Study Participants.

Summarized Questions	n (%)
Always eat breakfast before going to school	
Yes	305(37.2)
No	515(62.8)
Reasons attributed to missing breakfast before school	
Breakfast not prepared at home	123(23.9)
Parents give money to be used for buying food on way to school	178(34.6)
Fear of being late to school	133(25.8)
I prefer to buy food out from home as my breakfast	81(15.7)
Number of times skipped breakfast in past week	
Not skipped	305(37.2)
1-2 times/week	279(34.0)
3-4 times/week	130(15.9)
5-6 times/week	29(3.5)
7 times/week	77(9.4)
Number of times skipped lunch in past week	
Not skipped	491(59.8)
1-2 times/week	213(26.0)
3-4 times/week	69(8.4)
5-6 times/week	26(3.2)
7 times/week	21(2.6)
Number of times skipped supper in past week	
Not skipped	532(64.9)
1-2 times/week	195(23.8)
3-4 times/week	37(4.5)
5-6 times/week	21(2.6)
7 times/week	35(4.3)
Average number of cooked meals usually consumed per day	
1 meal	81(9.8)
2 meals	367(44.8)
3 meals	305(37.2)
4 meals	62(7.6)
>4 meals	5(0.6)

Table 2: Dietary Practices of Adolescents in Junior High Schools during the day.

Dietary practices of adolescents

Meal consumption and skipping behaviors: Table 2 shows the distribution of participants by frequency of meals consumption and meals skipping practices during the day.

Regarding consumption of breakfast, a large majority of the respondents 515(62.8%) indicated that they usually skipped breakfast served at home before going to school. When students were asked to indicate the number of times they skipped breakfast in the past one week prior to the study, 305(37.2%) reported that they did not skip breakfast throughout the week. Another 279(34.0%) skipped once or twice during the week; and 130(15.9%) skipped three or four times.

The common reason given by 178(34.6%) of the students who skipped breakfast was that their parents gave them money to buy food on their way to school. However, in most cases, but saved the money to browse on the internet after school. Another 133(25.8) indicated that they skipped breakfast because of the fear of getting to school late. Some 123(23.9%) also reported that breakfast was not usually prepared

Summarized Questions	n (%)
Food is usually brought from home to school	
Yes	276(33.7)
No	544(66.3)
Do you usually buy snacks/food at school during break time	
Yes	706(86.1)
No	114(13.9)
Type of food usually brought from the house to school	
Packaged or canned Fruit juice (eg. Kalyppo)	62(22.5)
Cocoa/Milo drink	11(4.0)
Pastries (eg. cookies, biscuits, cakes)	76(27.5)
Candies (toffee), chocolate	34(12.3)
Cooked food	93(33.7)
Type of snack/food usually bought in school	
Yogurt/fan ice, fan chocolate	112(13.7)
Soft drink (coke, fanta, sprite etc)	107(13.0)
Canned/packaged fruit juice (eg. Kalyppo)	96(11.7)
Fruit(eg. orange, banana, pineapple)	28(3.4)
Pastries (eg. cake, meat pie, sausage roll, doughnut)	135(16.5)
Cooked food (eg. Waakye[1], kenkey[2] and fish, fried rice and fried chicken)	342(41.7)
Who influences your decision on what you buy in school	
Parents	216(26.3)
Elder sibling	74(9.0)
Friends/class mate	82(10.0)
No one (I decide on my own)	448(54.6)
Snack preferences during the day	
Soft drink	287(35.0)
Ice cream/(fan ice, yoghurt)	208(25.4)
Pastries(meat pie, doughnut, cakes, cookies)	177(21.6)
Fruit (banana, orange, water melon, pineapple)	119(14.5)
No preference	29(3.5)
Frequency of soft drink consumption (no. of bottles consumed per week)	
None	277(33.8)
1-2 bottles/week	301(36.7)
3-4 bottles/week	163(19.8)
5-6 bottles/week	35(4.3)
>7 bottles/week	44(5.4)
Place where soft drink was consumed (n = 543)	
At School	287(52.9)
At Fast food setting/ restaurant/ supermarket/shop	164(30.2)
At Home	92(16.9)
Main reason for drinking soft drink (n = 543)	
Like flavour/taste	327(60.2)
Satisfy thirst	104(19.2)
Served or readily available at home	64(11.8)
Served at friend's house during a visit/party etc	48(8.8)

[1]Waakye: A combination of rice and beans mixed together and boiled
[2]Kenkey: Maize dough/corn dough mixed with water into a thick paste and boiled

Table 3: Information on Snacking habits and Food Consumption Practices during School Hours.

at home in the morning. The remaining 81(15.7%) preferred to buy breakfast on their way to school.

With regard to lunch, the majority 491(59.8%) ate lunch daily. Some of them (26.0%) skipped lunch once or twice in the previous week before the study. In addition 69(8.4%) and 26(3.2%) skipped 3 or 4 times per week and 5 and 6 times per week respectively. As in the case of lunch, the majority 532(64.9%) did not skip supper during the week. With respect to the average number of cooked meals usually consumed per day, a large number of the respondents 367(44.8%) indicated that they usually consumed two meals per day on the average; and less than half of the respondents 305(37.2%) usually consumed all three meals per day.

Information on snacking habits and foods consumption practices during school hours: Information regarding the snacking habits and food consumption practices of the respondents during school hours is presented in Table 3. When students were asked whether they usually brought food to school from their homes, the majority 544(66.3%) of them responded negatively. Of the 276(33.7%) students who usually brought food from home, 93(33.7%) brought cooked food such as boiled rice and stew/sauce. Another 76(27.5%) brought pastries (cookies, meat pie or cakes); and another 62(22.5%) brought canned/packaged fruit juice to school. A large majority 706(86.1%) of the students indicated that they usually bought a snack during break. Of the students who usually bought a snack or food at school, 41.7% indicated that they bought cooked food such as *waakye* (a combination of boiled rice and beans), *kenkey* (boiled corn dough) and fried fish, fried rice and fried chicken. Others 16.5% bought pastries; (13.7%), ice cream; 13.0%, soft drinks; and 11.7%, canned/packaged fruit juice. A majority (54.6%) of the respondents reported that they took their own decisions regarding what to buy in school. However, with some 26.3% of them, parents gave instructions as to what their children should buy. Other respondents 10.0% were influenced by friends or classmates or elder siblings as to the food that they bought during break.

When asked about their snack preferences (the type of snack they would prefer during the day), about a third (35.0%) of them preferred a soft drink for snack. Others 25.4% preferred an ice cream, 21.6%, pastries, and 14.5%, a type of fruit. When asked about the usual frequency of soft drinks consumption, 36.7% of the students reported that they drank between 3 and 4 bottles/cans during the week. Another 33.8% indicated that they drank between 1 and 2 bottles/cans per week. Only a few (5.4%) reported that they usually drank a bottle of soft drink every day in school.

When asked where they most often consumed soft drinks, the majority (52.9%) of the respondents indicated that they drank soft drinks most often at school. Another 30.2% stated that they drank soft drinks at a fast food setting, a restaurant, a supermarket or a shop. In a few cases (16.9%), soft drinks were readily available at home and at the disposal of the respondents to consume anytime.

The majority (60.2%) of the respondents who drank at least a bottle of soft drinks in a week indicated that they drank a particular soft drink because they liked the flavour or taste. Another 19.2% had a wrong perception that soft drinks could quench a person's thirst and therefore drank them to satisfy a thirsty feeling. Some 11.8% reported that they drank a soft drink in the week prior to the study simply because it was available at home at that time. With some 8.8%, the soft drink was served at a friend's house during a visit or a party.

Food preferences of respondents: To give an idea about the food choices that adolescents are likely to make, the students were required to select their preferences from a list of food items that had been prepared in different ways. Respondents were asked to select 12 food items they preferred or would usually be attracted to, out of 24 food items. The responses of the students regarding their food preferences

Food items	n (%)
Beverage	
Natural Fruit choice	228(27.8)
Soft drink/carbonated drink	592(72.2)
Egg	
Fried egg	445(54.3)
Boiled egg	375(45.7)
Vegetable salad preparation	
Vegetable salad with mayonnaise/salad cream	583(71.1)
Vegetable salad without salad cream/mayonnaise	237(28.9)
Preparation of chicken/meat	
Fried chicken/meat	645(78.7)
Roasted chicken/meat	175(21.3)
Type of Biscuit	
Creamy sandwiched biscuits/wafer	563(68.7)
Non-creamy sandwiched biscuits	257(31.3)
Type of Bread	
Sugar	536(65.4)
Brown/wheat	284(34.6)
Type of cooked yam	
Fried yam	436(53.2)
Boiled yam	384(46.8)
Type of Cooked Rice	
Boiled rice	337(41.1)
Fried rice	483(58.9)
Spread on bread	
Groundnut paste on bread	389(47.4)
Butter on bread	431(52.6)
Type of cooked plantain	
Fried plantain	592(72.2)
Roasted plantain	228(27.8)
Type of cooked corn	
Pop corn (sugar or salt)	612(74.6)
Boiled corn	208(25.4)
Breakfast beverage	
Coffee beverage	169(20.6)
Milo/cocoa beverage	651(79.4)

Table 4: Food Preferences of Study Participants.

are summarized in Table 4. A general observation made was that the majority of them had the tendency to opt for high fatty, sweetened and energy-dense foods. Regarding the intake of food items with high sugar content, for example, 72.2% preferred soft/carbonated drinks to natural fruit juices. Similarly, 65.4% preferred sugar bread to brown/wheat bread. Also, it was evident that, generally, most of the respondents preferred foods prepared by frying to those prepared by other healthier methods such as roasting or boiling which do not require the use of oil or fat. For example, the majority (78.7%) preferred fried chicken/meat to roasted chicken/meat. Similarly, a higher proportion (58.9%) of them preferred fried rice to plain boiled rice. Likewise, most of the surveyed students (72.2%) preferred fried plantain to roasted plantain.

Information regarding the differences between males and females with respect to some dietary practices is presented in Table 5. The Chi-square test statistics that was run indicated that there is no statistical significance difference between males and females with regard to whether the respondent ate breakfast or not before going to school. There was no statistical significance difference between males and females with respect to skipping breakfast, the average number of meals consumed daily and the number of soft drinks consumed weekly. However, there was a statistical difference between males and females with regard to the number of times that lunch and supper meals were skipped in the past week prior to the study. Generally, the findings revealed that most male adolescents usually skipped their lunch and supper meals as compared to females. There was also a statistical difference (between males and females with respect to their snack preferences during the day as indicated in Table 5.

Discussion

Addressing the increasing trends of overweight and obesity in the world remains a public health issue of great concern. This is because there is no evidence that the trends in the increasing cases of obesity are flattening off, let alone reversing. This study was conducted to assess the dietary practices and food preferences of Junior High School (JHS) children, because they are factors known to be associated with a person's weight status, and hence are potential causative factors of overweight and obesity in both children and adults. The majority 590(72.0%) of the respondents indicated that they often ate alone, compared with 176(21.5%) who often ate at table with their family members, 54(6.6%) reported that they often ate with their peers or friends. These findings are not encouraging, since they do not promote healthy eating patterns among children. It has been shown that when children eat together with their parents or other older family members at table, positive dietary practices are promoted among adolescents, to a greater extent [30,46-48].

The findings of this study showed that a majority of the school children did not always eat breakfast before going to school, consistent with the findings of previous studies which indicate that a typical habit of adolescents is skipping breakfast and meals in general [10,11,31,49,50]; and this habit has been reported to increase as they get older [51]. The reason given by most of the respondents for skipping breakfast before school was that they were given money to buy food on their way to school. A few other respondents said they were afraid of getting to school late or breakfast was never prepared at home. It has been reported that skipping breakfast has been employed as a means of saving time by most adolescents in the morning in order to get to school on time [11,52]. In addition, it has been reported that some adolescents skip meals, and in particular breakfast, in order to lose weight and maintain a slim body figure [52]. However, the implications of skipping breakfast are many. Some studies have shown that skipping breakfast is a potential cause of overeating at other meal-times, which could induce obesity and other lifestyle related chronic diseases [17,53,54]. For example, in a study involving a very large (N=10,000+) population-based sample of adolescents and their parents in Finland, the findings revealed that those who skipped breakfast most days of the week were significantly more likely to become overweight or obese than the regular breakfast eaters [55]. Similarly, other large (N=8000+) studies conducted in the United States have also shown that breakfast skippers are more likely to become overweight [53,54], or worse, become obese [56].

Several studies consistently relate consumption of breakfast to improvements in academics (test scores and grades) [57-59]. For example, in a study involving a sample of 6,463 teenagers from Korea, it was reported that teenagers who skipped breakfast regularly scored lower marks compared with the regular breakfast eaters [57]. Similarly, another study undertaken in Saudi Arabia [60], involving a sample of 800 students (aged 9-21) revealed that regular breakfast skippers had significantly lower school grades than regular breakfast eaters. Breakfast consumption has also been shown to improve alertness, mood, word recall, memory, cognition, physical and mental performance, and reduce behaviour problems [58,61].

Question item summarized	Males n(%)	Females n(%)	Statistics
Always eat breakfast before going to school			Pearson Chi-square = 1.299
(yes= 305)	186(61.0%)	119(39.0%)	Sig (*p*- value) = 0.250
Always eat breakfast before going to school	299(58.0%)	216(42.0%)	df=1
(no =515)			
Number of times skipped breakfast in past week			
Not skipped	193(63.3%)	112(36.7%)	Pearson Chi-square = 3.045
1-2 times/week	155(55.6%)	124(44.4%)	Sig (*p*- value) = 0.550
3-4 times/week	75(57.7%)	55(42.3%)	df=4
5-6 times/week	19(65.5%)	10(34.5%)	
7 times/week	43(55.8%)	34(44.2%)	
Number of times skipped lunch in past week			
Not skipped	263(53.6%)	228(46.4%)	Pearson Chi-square = 13.257
1-2 times/week	146(68.5%)	67(31.5%)	Sig (*p*- value) = 0.010
3-4 times/week	47(68.1%)	22(31.9%)	df=4
5-6 times/week	16(61.5%)	10(38.5%)	
7 times/week	13(61.9%)	8(38.1%)	
Number of times skipped supper in past week			
Not skipped	287(53.9%)	245(46.1%)	Pearson Chi-square = 14.784
1-2 times/week	128(65.6%)	67(34.4%)	Sig (*p*- value) = 0.005
3-4 times/week	30(81.1%)	7(18.9%)	df=4
5-6 times/week	16(76.2%)	5(23.8%)	
7 times/week	24(68.6%)	11(31.4%)	
Average number of cooked meals usually consumed per day			
1 meal			
2 meals	49(60.5%)	32(39.5%)	Pearson Chi-square = 5.462
3 meals	207(56.4%)	160(43.6%)	Sig (*p*- value) = 0.243
4 meals	191(62.6%)	114(37.4%)	df=4
>4 meals	35(56.5%)	27(43.5%)	
	3(60.0%)	2(40.0%)	
Snack preferences during the day			
Soft drink	175(61.0%)	112(39.0%)	Pearson Chi-square = 19.280
Ice cream/(fan ice, yoghurt)	128(61.5%)	80(38.5%)	Sig (*p*- value) = 0.004
Pastries(meat pie, doughnut, cakes, cookies)	98(55.4%)	79(44.6%)	df=4
Fruit (banana, orange, water melon, pineapple)	67(56.3%)	52(43.7%)	
No preference	17(58.6%)	12(41.4%)	
Frequency of soft drink consumption (no. of bottles consumed per week)			
None			
1-2 bottles/week	152(54.9%)	125(45.1%)	Pearson Chi-square = 8.143
3-4 bottles/week	185(61.5%)	116(38.5%)	Sig (*p*- value) = 0.086
5-6 bottles/week	101(62.0%)	62(38.0%)	df=4
>7 bottles/week	20(57.1%)	15(42.9%)	
	27(61.4%)	17(38.6%)	

Table 5: Comparison between Males and Females with respect to Dietary Practices.

It has also been indicated that even a short-term lack of breakfast can lead to a reduction in concentration, difficulties with recalling new information, and verbal fluency [58].

Regarding intake of carbonated soft drinks, approximately 66% of the study population reported that in the previous one week prior to the study they drank at least one bottle or can of a soft drink. Consumption of soft drinks merits some special consideration as a dietary habit of adolescents, because soft drinks, like other sugar-sweetened beverages, have a very high energy density due to their high sugar content, which when taken in excessive quantities, can increase one's susceptibility to become overweight or obese [62-64]. Ironically, in another study it was found out that soft drinks consumption is one of the behaviours adopted by adolescents for the prevention of excessive weight gain, suggesting that some adolescents had a wrong perception and were ignorant that drinking soft drinks does not prevent excessive weight gain [65]. Similar studies which assessed the snacking habits of adolescents also reported a high prevalence of soft drinks intake among adolescents [24,34,66,67]. The effects of consuming soft drinks in excessive quantities are manifold. One effect of consuming soft drinks frequently is their ability to displace the micronutrients present in nutrient-dense beverages such as milk and fortified fruit juices [68-70]. Other studies all reported that adolescents tend to replace milk with soft drinks, both during meals and throughout the day [20,21,68-70]. It has also been reported that soft drinks consumption is significantly correlated with

the severity of dental erosion [71] which is mainly a result of the high concentrations of sugar and acids present in soft drinks which easily dissolve the tooth enamel.

The major reason why the respondents drank soft drinks was because they liked its taste and flavour. In similar studies, the pleasant flavour and taste of soft drinks was mentioned as the main reason which led to the high consumption by adolescents [22-24]. It has also been reported that most adolescents choose their diet on the basis of taste rather than nutrition [72]. In the present study some students reported that they drank soft drinks in order to quench their thirst which has also been reported in similar studies [23,24].

Most of the respondents reported that the school setting is the most common setting in which soft drinks is consumed regularly. In the school environment, soft drinks are readily available and sold in an attractive manner – in school canteens and shops which encourages the consumption of these beverages [24,73]. For example, in a survey conducted by Fernandes [73] in 2,023 schools in the United States, it was found out that soft drinks were available at cafeterias or from vending machines in 40% of these schools. In the present study, the home was the second most common setting for the consumption of soft drinks. Grimm and his colleagues [70] also found out that intake of soft drinks among school-aged children was very highly correlated with taste preferences, availability in the home and school settings. Therefore, it was recommended in a study that parents and the home environment are important potential intervention targets which can help address the issue of excessive soft drinks consumption among adolescents [74]. In addition, the WHO global strategy on diet, physical activity and health suggests limiting access to unhealthy foods and soft drinks sales at schools [75].

When the study participants were asked about their snack preferences, the majority indicated that they preferred a soft drink for snack during the day. Others preferred ice creams and pastries. These food items are high-caloric, energy-dense foods containing high quantities of sugar and fats which increase the risk of becoming overweight and obese or suffering from a chronic disease like diabetes, later on in life.

Another noteworthy finding is that only a few of the respondents indicated that they preferred a fruit or usually bought a fruit for snack while in school. Similarly, other studies have reported that most adolescents do not usually eat fruits and vegetables on a daily basis and so are unable to meet the World Health Organization (WHO) goal of a daily intake of at least 400 grams of fruit and vegetables [76-79].The findings of this study suggest that, maybe, fruits are not always available to be bought and consumed by students. Another inference that can be drawn is that parents may also not be providing fruits often at home perhaps because of their high prices, evident from the finding that most adolescents are often not given fruits to take to school. In a review paper, evidence from 98 quantitative studies on fruits and vegetables intake among children and adolescents revealed that the determinants for high consumption levels of fruits and vegetables among adolescents include high socioeconomic position (SEP), high parental intake of fruits and vegetables and high availability and accessibility of fruits and vegetables at home [80]. The findings of some other studies also reveal that, among adolescents, taste is a main reason for not liking fruits and vegetables, especially vegetables [81-84].

Conclusions

In conclusion, this study revealed that Ghanaian adolescents in Junior High Schools are practicing various unhealthy eating habits which include meal skipping, particularly breakfast, snacking daily on high-content fatty and energy-dense foods, such as soft drinks and pastries during school hours. It is imperative that frantic efforts are made to help adolescents cultivate an interest in eating healthy foods and developing healthy dietary practices. This will go a long way to help reduce the incidence of diet-related health conditions such as hypertension, stroke, and diabetes, the prevalence of which is increasing in Ghana, especially during the later stages of adulthood.

Recommendations

Interventions to reduce the consumption of soft drinks should target availability in both the home and school environments, by limiting the sale of soft drinks and replacing them with more nutritive beverages such as natural fruit juices. Food vendors on school premises should be encouraged to provide at affordable prices a wide variety of healthy foods such as appealing fresh fruits and vegetables for students to buy. Also, it would be very prudent to include lessons on healthy nutrition (with practical aspects) in the curriculum of Primary, Junior High and Senior High Schools, as has been done in some developing countries [48,85,86].

There is also a need for further studies to obtain the views of school children on factors (barriers and promoters) at home and in school which affect their desire to eat healthy foods.

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Nutritional Regulation for Meat Quality and Nutrient Metabolism of Pigs Exposed to High Temperature Environment

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Abstract

High ambient temperatures not only affect animal production but also pig welfare. The decline of the production performances and meat quality of pigs during heat stress were traditionally considered to a cause of the decreased feed intake. However, it recently has been shown that heat stress disturbed the nutrients metabolism including protein, lipid and carbohydrate, and made the body reorganizes the utilization of the nutrition. High temperature also disturbed the cell function and antioxidant system. This paper reviewed the effect of heat stress on growth performance, meat quality and nutrients metabolism of pigs and their nutritional regulation for meat quality.

Keywords: Meat quality; Nutrient metabolism; Heat stress; Nutritional regulation; Growth performance

Introduction

According to the Food and Agriculture Organization of United Nations (FAO) statistics, over 50 percent of pig industries were in tropical and subtropical regions. The long-term hot weather of these regions would have a greater negative effect on pig production, especially with the development of intensive high density production, the effect of high temperature stress on pig production has become more and more serious, not only affecting the pig production performance, meat quality, but also affecting the sustainable development and economic benefits of the pig industry. In this paper, the effects of heat stress on pig production performance, meat quality and its molecular mechanism, and the nutritional regulation methods are reviewed, those results provide theoretical basis for reducing the effect of heat stress on the production and meat quality of pigs.

Effect of heat stress on growth performance of pig

Because the sweat gland of the pig is not developed, pigs maintain the body temperature mainly through breathing and stretching in the high temperature environment. the suitable environmental temperature of finishing pigs is 10-23.9°C, when the ambient temperature is higher than 24°C, finishing pigs begin cooling through breathing, and when the temperature continue to rise, the frequency of pigs lying on the ground increase and their activity decrease [1,2].

Le Dividich et al. [3] reported that when the environmental temperature was between 20°C and 30C, the feed intake decreased by 40-80 g per day with the temperature increasing by 1°C. The sensitivity of pig to the environment temperature in different stages is different, usually the effect of high temperature on the feed intake and daily gain of pigs increased with the increase of pig weight. Ai et al. [4] found that under high environmental condition (28-35°C), the daily feed intake of pigs in the phase of 15-30 kg, 30-60 kg and 60-90 kg decreased by 9%, 41% and 20%, and the daily gain decreased by 9%, 21% and 23% respectively. Guo et al. [5] studied the effects of different environmental temperature (23°C, 26°C, 29°C, 32°C and 3°C) on the daily feed intake of pigs at different weight stages (40-60 kg, 60-80 kg and 80-100 kg), and found that when the temperature rose, the feed intake decreased linearly with the temperature increasing from 23 to 32°C, the heavier the body weight, the greater the effect of temperature. When the temperature was over 32℃, the daily feed intake decreased by 101 g with the temperature increasing by 1°C.

Quiniou et al. [6] reported that the daily feed intake of 60-90 kg pig decreased by 128 g with the temperature increasing by 1℃ under the 22-29°C condition. Le Bellego [7] also found that the daily feed intake of 65-100 kg pig decreased by 78 g with the temperature increasing by 1°C under 22-29°C condition. Shi et al. [8] reported that the daily feed intake of 80-100 kg pig decreased by 116 g with the temperature increasing by 1°C under 24-35°C condition, the daily weight gain also decreased significantly. These results above were confirmed by Yang et al. [9].

In conclusion, the effect of environmental temperature on the growth performance of pigs was related with feed intake, but the relationship between the environmental temperature and the feed intake of pigs was different for the different temperature, feeding time and pig weight, it need to be analyzed according to the specific circumstances.

Effect of heat stress on meat quality

Meat quality also is influenced by environmental temperature. A large number of studies have reported that heat stress before slaughter stimulated the catecholamine secretion in finishing pigs, which caused muscle glycogen rapid decomposition, producing large amounts of lactic acid, the muscle pH decrease and PSE meat formation [10]. Yang et al. [9] reported that continuous high temperature significantly reduced the pH of longissimus muscle at 24 h postmortem, increased

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the drip loss at 48 h postmortem and shearing force of longissimus muscle. The environmental temperature in summer is usually higher than in winter, and the meat quality also is inferior to in winter [11]. High temperature also decreased the intramuscular fat content and increased the L^* value of the muscle in finishing pigs [12], but not in growing pigs [13], because the content of intramuscular fat in growing pigs is vey low. Le Dividich et al. [3] indicated that the fat deposition in muscle of finishing pig decreased significantly under high temperature condition, which may be related to the decrease of feed intake and energy consumption. Lefaucheur [14] found that compared with growing pigs less than 10C temperature condition, the metabolism of muscle fibers of semitendinosus muscle in growing pigs under 28°C condition changed obviously, the fiber types also changed accordingly. High temperature increased the sugar fermentation potential and changed the energy metabolism of the muscle fiber, with the change of the muscular fiber type [15]. Using C_2C_{12} cell line as research model, it was found that the sustained high temperatures induced muscle cells differentiation from fast to slow transformation [16]. Shi [8] reported that high temperature increased the MyHCIIx fiber and decreased the MyHCIIb fiber ratio. While in Mardies et al. [17] studies, higher temperature decreased the MyHCIIb gene. The reason for this difference may be due to the tolerance ability of the pig to heat stress, the setting of the environmental temperature and the duration of the treatment.

Effect of high temperature on nutritional metabolism of pigs

In acute or chronic heat stress conditions, animals reduced their feed intake by improving leptin, adiponectin and its receptor expression [18,19]. Leptin activated the hypothalamus axis, resulting in reduced feed intake [20], adiponectin also regulated feed intake through central and peripheral nervous system [21]. In addition, heat stress reduced feed intake and nutrient digestion and absorption partly through affecting insulin, cortisol and ghrelin secretion [22,23,8].

High temperature also decreased the body protein metabolism and deposition. When the environmental temperature reached 30°C, the amount of body protein deposition in growing pigs decreased directly or indirectly by the decrease of feed intake [7]. Kerr [24] also reported that high temperature (33°C) reduced the content of pig carcass protein. Short term heat stress increased protein degradation, reduced protein synthesis and retention; it also decreased the plasma level of aspartic acid, serine, tyrosine and cysteine in finishing pigs [25]. While long-term heat stress blocked protein synthesis, reduced the protein decomposition and amino acids level in blood (especially sulfur and branched chain amino acids), and increased the plasma level of aspartic acid, glutamic acid and phenylalanine [26,27]. Long-term heat stress reduced protein catabolism might by activating gluconeogenesis pathway, which increased the glucose level to provide more energy and reduce protein usage.

Long term heat stress also decreased the body lipid metabolism and fat distribution; the fat had a trend to transfer from the outer layer to the inner [3], which is more benefit for the body heat dissipation. Kouba [28] found that the high environmental temperature increased the activities of LPL in longissimus muscle and periphery kidney by 144.6% and 90.5%, respectively. Rinaldo and le Dividich [29] also found that high temperature reduced the content of back fat, the activities of malate dehydrogenase (ME) and glucose-6-phosphate dehydrogenase (G-6-PDH) in back fat and periphery kidney fat by 60%. Wu et al. [30] reported that high temperature (33°C) had a trend to decrease the intramuscular fat of longissimus muscle in finishing pigs, it also inhibited the fatty acids de novo synthesis ability by decreasing the activities of acetyl coenzyme A carboxylase enzyme (ACC), high temperature also decreased the content of FAS in the longissimus muscle and beta oxidation of fatty acids in skeletal muscle by decreasing the activity of L (+) P-hydroxyacyl CoA dehydrogenase (HAD). In addition, high temperature decreased the activity of lipolytic enzymes [26] and the level of non-esterificated fatty acids (NEFA) [31], which was independent of the reduction of feed intake. Under high temperature condition, the activity of lipolytic enzymes in adipose tissue decreased to limit heat production for adaptation to high temperature environment.

Carbohydrates could easily be converted into intermediate metabolites to generate ATP. The formation of ATP was regulated by three major signaling pathways: glycolysis, tricarboxylic acid cycle and oxidative phosphorylation. Mild heat stress activitied muscle glycogen phosphorylase and pyruvate dehydrogenase, but did not affect the content of glucose 6-phosphate, lactic acid, pyruvic acid, acetyl CoA, creatine and phosphocreatine and ATP [32]. Chronic heat stress reduced the plasma level of glucose, made more glucose enter the tissue from the blood to supply the energy, then the fat supplying energy decreased. As feed intake and blood glucose decreased, the glucose meet the need of the production decreased, so the energy in heat stressed pigs need to be rebalanced.

Effect of heat stress on cell and its stress protein

Heat stress interfered with the balance between oxidation and anti-oxidation in cells, resulting in excessive production of free radicals, damaging the body defense function [33], even producing cytotoxicity [34]. Heat stress also induced iron releasing from ferritin and reacting with H_2O_2 to produce ferric oxide ion, which was one of the important reasons for heat stress causing dark color of meat [35,36]. In addition, ROS was produced from NADP+ transformed to NADPH [37] by NADPH oxidase under high temperature condition. Shi [8] also found that high temperature enhanced the activity of NADPH oxidase. Moon et al. [38] reported that high temperature activated NADPH oxidase and increased the NADP+/NADPH ratio, NADPH prevented the biosynthesis of NADP+, acting as a cytochrome C reductase inhibitor, indicating that heat stress decreased the oxidation resistance of the body by destroying the oxidative respiratory chain.

Heat stress has cellular toxicity, because it disturbs the biological function and metabolism of the cell, leading to oxidative damage, and even apoptosis necrosis of the cells [39]. Although ROS was produced mainly from mitochondria, it first damaged mitochondria by disrupting oxide complex I, II, V and IV, resulting in destruction of respiratory chain and reduction of ATP [40]. In rodent animal, the morphology and structure of mitochondrial changed under high temperature condition, oxidative phosphorylation and energy production decreased, which could not meet the needs of cell metabolism, the mitochondrial membrane lipid also was oxidized and mitochondrial protein was degraded [41,42]. Under heat stress condition, free radicals activated the intrinsic apoptotic signaling pathway based on the mitochondrial pathway of mitochondrial membrane [43], once released, the cytochrome c moved to the cell fluid, activating the effective factor of apoptosis proteins (called apoptosis protease), resulting in programmed cell apoptosis. Although cell function and protein synthesis were disturbed under heat stress conditions, heat stress proteins synthesis were stimulated, including HSP 110, HSP 100, HSP 90, HSP70, HSP 60, HSP 40 and HSP 10. As heat stress proteins acted as molecular chaperone and contributed to the protein synthesis, folding, assembly, transportation and degradation of thermal stress degradation [44]. Among these heat stress proteins, HSP70 and HSP

90 were the most important [45]. Shi [8] also found that heat stress increased the expression of HSP70 gene in pig muscle. Heat stress damaged many proteins, while heat stress proteins helped to repair them. Heat stress also influenced the fluidity and stability of the cell membrane and inhibited the function of transporter protein [46] on the cell membrane. When the ambient temperature elevated from 25°C to 35°C or above, heat stress activated sphingomyelinase, phosphatase and phosphatidylinositol phosphate kinase [47], stimulating the accumulation of phosphatidic acid (PA) and phosphatidyl inositol (bisphosphate (PIP2). PIP2 is a signal molecule of fat, which delayed the activation of upstream signal of cell repair function. Under heat stress condition, the structure of the lipid material is decomposed to produce PA [48], which inserted into different cell membrane sites and decreased the cell membrane fluidity. The concentration of Ca2+, Na+ and K+ ions in the cells also increased with the decrease of cell stability [49] and destroy of the ion channels.

Nutritional regulation of improving meat quality

Protein and amino acids regulation: Under high temperature condition, decreasing dietary protein levels with balanced amino acids was one of the important methods alleviating the heat stress to pig, which did not reduce the pig net energy intake and production performance. Stahly et al. [50] reported that supplementation of synthetic lysine instead of natural protein in favor of pig production performance. Le Bellego et al. [51] reported that decreasing the dietary protein level of growing pigs and finishing pigs under high temperature condition reduced the pig protein deposition, but fed low protein level diet with balanced ideal amino acid pattern didn`t affect pig growth performance and carcass traits. Peng et al. [52] concluded (according to current literature review) that the appropriate dietary protein level of growing pigs under high temperature conditions was from 14% to 16%, with the equilibrium model of amino acid lysine, threonine, methionine, tyrosine, isoleucine, leucine, valine to dietary ratio was 1.02%, 0.74%, 0.29%, 0.23%, 0.85%, 1.62% and 0.96% respectively; the appropriate dietary protein level of growing pigs under high temperature conditions was from 12% to 13%, with the equilibrium model of amino acid lysine, threonine, methionine, tyrosine, isoleucine, leucine, valine to dietary ratio was 0.84%, 0.58%, 0.27%, 0.17%, 0.53%, 1.09% and 0.66% .

Supplementation with anti-stress agents

Supplementation with vitamin C, vitamin E or niacin: Pigs were nervous and their glucocorticoid secretion increased under heat stress condition, supplementation with vitamin C reduced glucocorticoid secretion and improved the ability to resist stress of pigs. Frei et al. [53] reported that vitamin C effectively resisted the active oxygen free radicals and prevented the damage of the biological membrane from the oxides. Vitamin C also was the most important antioxidants and improved the meat quality of pigs [54]. In order to reduce the occurrence of PSE meat, the content of vitamin C in the diet should be more than 50 mg/kg. Vitamin E played an important role in the stability of lipid, improving pork color and reducing water loss of pork [55,56]. Cheah et al. [57] showed that when vitamin E in the pig diet was higher than 500 mg/kg, it significantly decreased the drip loss of meat; when vitamin E was above 1000 mg/kg, the release of Ca2+ and the occurrence of PSE meat were significantly decreased. Buckley et al. [58] reported that dietary vitamin E increased from 10 mg/kg to 100-200 mg/kg, the quality of pork was positively correlated with the dose of vitamin E. Real et al. [59] reported that Niacin increased the pH value, reduced the drip loss and L value of longissimus muscle.

Regulation of electrolytes: The potassium and carbonate excretion increased, and the sodium and hydrogen discharge decreased in pigs under high temperature condition, which affected the mineral balance. Potassium chloride, chloride or sodium bicarbonate electrolyte should be added to the pig diets appropriately to reduce the damage caused by heat stress [60]. Supplementation of 0.1 to 0.2% sodium bicarbonate or 0.1 to 0.2% vitamin E to the pig diets had good effect on preventing heat stress. Wu et al. [61] found that compared with the control (under the heat stress condition), the average daily weight gain of piglets who drinking the electrolyte solution containing sugar (containing sugar, sodium chloride, potassium chloride, sodium bicarbonate, citric acid et al.) was significantly increased by 15.64%. Feng et al. [62] reported that the average daily gain of pigs was significantly increased by 5% with supplementation of 0.3% sodium bicarbonate, 0.2% potassium chloride and 0.02% chromium nicotinate respectively to the diets of finishing pigs under the high temperature condition. Ao et al. [63] reported that in the summer heat stress conditions, the average daily feed intake of finishing pigs who drinking 2% electrolyte balance agent at the first 5 days and drinking 1.25% electrolyte balance agent at the last 25 days increased by 10.14%, the average daily gain increased by 95 g and feed gain ratio decrease by 9 %, and there were no obvious thermal stress clinical indications of the pigs.

Supplementation with trace elements or minerals: Zhang et al. [64] supplemented 300 g/kg chromium picolinate to the diets of pigs under high temperature condition, the average daily gain and average daily feed intake was not different from the control group at the first two weeks; while the average daily feed intake increased by 10.2%, the average daily gain increased by 38.1% at the last 2 weeks, the chromium metabolism also was improved. Wang [65] reported that supplementation of 530 mg/kg zinc methionine and 200 mg/kg pyridine chromium carboxylate to pig diets increased the average daily feed intake and the average daily gain and decreased the feed gain ratio under the high temperature condition, increased the protein decomposition and gluconeogenesis, and improved the production performance. Liu et al. [66] reported that selenium (Se) had a synergistic effect with CAT and SOD in the removal of lipid peroxide, it also played antioxidant role coordinated with vitamin E. Mahan et al. [67] reported that the drip loss of dorsal muscle decreased by supplementation with 0.1 mg/kg Se and a certain amount of VE and VC. Torrent [68] reported that 0.3 mg/kg selenium (Se) in the growing pig diet reduced the occurrence of PSE meat. Alonso et al. [69] found that high level of magnesium (Mg) increased the pH value and decreased the speed of the glycogen, slow down the pH value and reduced the occurrence of PSE meat. Peeters et al. [70] reported that supplementation with Mg reduced stress, improved the muscle system hydraulic. Manganese also improved the meat quality of pigs.

To sum up, the production performance of pigs is affected by the reduction of feed intake, which is caused by the influence of the pig's nervous system under heat stress condition. The synthesis of protein, fat and carbohydrate also is affected by the heat stress. Under heat stress condition, pigs how to re-balance the protein, fat and carbohydrate metabolism to adapt the environment need to be further studied. In addition, heat stress affects the body's antioxidant system directly through the ROS, leading to the damage of cell function and decrease of the meat quality, but till now, there are no reports about the relationship between the environment temperature and PSE meat formation. Whether high temperature causing PSE meat just through ROS production also need to be further researched. Nutrients adjustment can reduce heat stress, slow down the oxidation and improve pork quality, which is also important for human health. In

the further, more effective anti-heat stress additives need to develop to meet the need of pig production.

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Performance, Immunity, Meat Composition and Fatty Acid Pattern in Broilers after Dietary Supplementation of Fermented *Ginkgo biloba* and *Citrus junos*

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Abstract

Dietary Fermented *Ginkgo biloba* (FGLP) and *Citrus junos* Probiotics (FCJP) was evaluated on growth performance, immunity, cecal microbiology, meat composition and fatty acid profile in broilers. A total of 150 one day old Ross 308 broilers were randomly allocated to the 5 treatments (5 replicated pen of 6 birds/pen): 1) Control (corn-soybean meal based basal diet), 2) FGLP1: Corn-soybean meal based basal diet+5% FGLP, 3) FGLP2: Corn-soybean meal based basal diet+10% FGLP, 4) FCJP1: Corn-soybean meal based basal diet+5% FCJP, and 5) FCJP2: Corn-soybean meal based basal diet+10% FCJP. A significant increase in weight gain during starter (0-21 days), finisher (22-35 days) and overall period (0-35 days) was exhibited in FGLP2 supplemented group compared to control and other treatment groups ($P<0.05$); where better feed conversion efficiency was found after FCJP2 supplementation during finisher and overall period compared to control and other treatment groups ($P<0.05$). Serum IgG was elevated in FGLP and FCJP supplemented group relative to control birds ($P<0.05$). Meat composition data elucidated that, thigh meat crude protein and crude fat content was increased in FCJP1 supplemented group compared to control ($P<0.05$). There was found no significant differences in meat cholesterol content between control and treatment groups ($P>0.05$); however, among meat fatty acids, sum of SFA in thigh meat was diminished in FGLP group and sum of MUFA in breast meat was diminuted in FGLP and FCJP group compared to control ($P<0.05$). Sum of n-3 PUFA of breast and thigh meat was elevated after supplementation of FGLP and FCJP compared to control ($P<0.05$). To sum up, dietary FGLP and FCJP supplementation significantly improved performance and immunity, decreased SFA and elevated n-3 PUFA of broiler meat. Therefore, FGLP and FCJP probiotics could be supplemented as functional feed additives in broilers diet.

Keywords: *Ginkgo biloba* probiotics; Fermented *Citrus junos* probiotics; Growth performance; Immunity; Meat quality

Introduction

Since 5000 BC aromatic plants or medicinal plants (herbs and spices) have been used in the Middle East for their medicinal properties, preservative capabilities, aroma and flavor enhancing in the food and food products [1]. Due to their beneficial properties, according to World Health Organization (WHO) around 80% of the total global populations (especially in the developing countries) depends on the medicinal plants based medicines for their health care [2,3] On the other hand, antibiotic growth promoters were utilized since 1950s for long time in animal production for allowing adequate productivity [4]; however due to their antimicrobial resistance in humans it was banned in the European Union on January 1st 2006, and then all over the world it becomes an important issue of banning in the animal production [5,6]. Therefore, the aromatic plants attracted by the researchers as alternative source for the animal production and health, as they are being utilized for long in the human medicines and food products. Different the aromatic plants, their extracts, essential oils tested as natural feed additives found to be advantageous as an alternative to antibiotic growth promoters, and they are considered as the generally recognized as safe and are residue free [7]. The feed additives derived from the natural plants are known as the botanicals or phytogenics or phytobiotics which can be utilized in the animal's diet to promote the performance and quality of the resulted feed and animal products [5,6,8].

Aromatic plants are found all over the world as wild or in some case as cultivated, all of them composed of chemical substances which are called primary and secondary metabolites such as flavonoids, polyphenols, polypeptides, alkaloids [9,10]. These bioactive compounds can act as therapeutics with having antioxidant and antiseptic properties [11]; antimicrobials and can contribute in the microbial growth retardation activities on the food and food products [12,13]; helps is inhibiting the oxidative rancidity and delaying the off-flavour development in food products [14,15]. The term probiotics become very common both for human and animal study; where the word "Probiotics" actually derived from the Greek word and meaning is 'for life' ("Antibiotic" means 'against life') and denoted the beneficial microorganisms and tested as single or combined state for promoting the growth performance in animal and poultry nutrition [5,16,17], immune enhancer through enhancing T-cell function and antibodies, antigenic stimulation by the secretion of immunoglobulin [18,19]. Fermentation of feed or agricultural byproducts can enhance nutritional quality of the product and able to improve the performance of animals and reduce cost in broiler [8,20]. Fermentation of plant materials along with probiotic can improve performance in broilers and cattle [21,22] and no adverse impact on broiler performance [23]. The benefit of utilizing natural plants or their products along with

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fermentation is cost-effective, environmental friendly and performance enhancer [8,24]. Fermentation of plant materials (*Punica granatum*, *Ginkgo biloba, Camellia sinensis, Alisma canaliculatum*) and multi-microbe probiotics reported that it can enhance the performance as well as the meat quality in broilers [9,21-24].

Several types of researches are going on all over the world to get more efficacies through utilization of probiotics and natural plant materials in poultry nutrition as well as in animal nutrition. The single or multi-microbial addition in basal diet; single or multi-plant material addition in basal diet; or combination of probiotic organism along with natural plant material, in single or multi-state dimension could be the research interest to test the efficacy in animal and poultry nutrition. Kim et al. [25] reported that a combinations of multi-plant materials along with multi-microbe fermented product exhibited better productive performance in case of broiler; where Bostami et al., [26] conducted a combination of two plant material along with multi-microbe fermented product in case of broiler, where both the performance and meat quality was improved in case of broiler. Since there is a huge amount of natural plants on the planet, the estimated amount is 70,000 species of folk medicine reported by Farnsworth and Soejarto [27], and around 21,000 plant taxa utilized [28] for medicinal purpose reported by WHO [29]. Different plant materials are composed of different secondary metabolites while the probiotic microorganisms also possess different organic substances which can exhibit synergistic actions in the poultry and animal nutrition [25,29,30]. Among different plant materials utilized for the human and animal medicinal purpose in Korea, China and Japan, *Alisma canaliculatum*, *Houyttunia cordata*, *Camelia sinensis*, *Citrus junos*, *Ginkgo biloba*, *Laminaria cordata*, *Salicornia herbacea, Glycyrrhiza glabra* and so on are available and under research investigation. Where *Ginkgo biloa* is under the family of *Ginkgoaceae*; and *Citrus junos* is under the family of *Rutaceae*; *Punica granatum* is under the family of *Lythraceae* are composed of different bioactive compounds which are tested as single state in different separate studies with positive or neutral impact in poultry [24,25,31]. To the best of our knowledge, there is no study conducted yet on combination of these plant materials along with fermentation of multi-microbe probiotics.

Therefore, in this study, the purpose is to prepare two fermented products using the plant materials and probiotics and then compare that two fermented products with the control diet. The fermented products were as follows: 1) FGLP: Combination of *Ginkgo biloba+Punica granatum* and fermentation with *Lactobacillus plantarum, Lactobacillus acidophilus* and *Saccharomyces cerevisiae;* 2) FCJP: Combination of *Citrus Junos+Punica granatum* and fermentation with *Lactobacillus plantarum, Lactobacillus acidophilus* and *Saccharomyces cerevisiae.* Where fermented probiotic product was prepared using two level (5% and 10%) of *Ginkgo biloba* and *Citrus Junos* and then added at the rate of 0.4% of the total basal diet in case of broiler. As a continuation of our target of development of functional feed additives by the proper utilization of natural plant materials with the beneficial microorganisms through testing the efficacy in broiler, pigs and cattle, and to suggest the farmers; the objective of the present study is testing the efficacy of fermented *Ginkgo biloba* and *Citrus Junos* probiotic in broilers on their performance, immunity, cecal microbiology, meat composition and meat fatty acid profiles.

Materials and Methods

Preparation of fermented *Gingko biloba* and *Citrus junos* probiotics

Gingko biloba leaf and *Citrus junos* rind was obtained from Boseong, Republic of Korea. *Punica granatum* byproduct, which is a Goheung-gun cultivar, was collected from a juice manufacturing company. The byproduct was composed of about 80% peels and rinds and 20% seed. *Gingko biloba* leaf, *Citrus junos* rind and *Punica granatum* were then dried in a forced air oven (Doori TEC, Doori TEC, FA, Co., Ltd.) at 80°C for 3 days and subsequently ground into powder that could pass through a 0.15 mm sieve using a milling machine [32]. Samples were then tightly packed in polythene plastic bags, after which they were sealed and kept at room temperature until needed. *Gingko biloba* leaf, *Citrus junos* rind and *Punica granatum* skin were analyzed in triplicate for Crude Protein (CP), Ether Extract (EE), moisture and ash as described by the Association of Official Analytical Chemists [33]. The fatty acid composition was determined by a direct method for Fatty Acid Methyl Ester (FAME) synthesis using a Gas Chromatograph (GC). The pH was measured using a digital pH meter (Docu-pH+meter, Sartorius, USA).

Fermented *Ginkgo biloba* Probiotics (FGLP1) contains 65% defatted rice bran, 30% pomegranate peel extract, and 5% *Ginkgo bloba* leaf powder; Fermented *Ginkgo biloba* Probiotics (FGLP2) contains 60% defatted rice bran, 30% pomegranate peel extract, and 10% *Ginkgo bloba* leaf powder. Whereas Fermented *Citrus junos* Probiotics (FCJP1) contains 65% defatted rice bran, 30% pomegranate peel extract, and 5% *Citrus junos* rind powder; Fermented *Citrus junos* Probiotics (FCJP2) contains 60% defatted rice bran, 30% pomegranate peel extract, and 10% *Citrus junos* rind powder. After mixing the ingredients to prepare FGLP and FCJP, samples were inoculated with 30% (v/w) *Lactobacillus plantarum* KCTC 3099 and *Lactobacillus acidophilus* KCTC 3111 and fermented for 2 days at 37°C and 40% moisture in a commercial fermenter (W-1000; Wonbalhyo Industry Co., Incheon, South Korea). After fermentation the medium was again inoculated with 30% (w/v) *Saccharomyces cerevisiae* KCTC 7904 and fermented for 3 days at 37°C. Fermentation with microbial inoculum was conducted using a cycle of 5 h standing and 3 h shaking to ensure proper mixing and fermentation. Subsequently, the fermented sample was dried in a forced air oven (Doori TEC, Doori TEC, FA, Co., Ltd.) at 32°C for 2 days to reduce the moisture levels. During fermentation with microbial inoculum, there was a cycle of 5 h standing and 3 h shaking to obtain the proper mixing and fermentation. Finally, FGLP and FCJP were stored in an air-tight plastic bag until being mixed with basal diet. The microbial concentration, proximate composition, trace minerals, fatty acids and pH of FGLP and FCJP were analyzed in triplicate. To determine the number of cells, 1 g of FGLP and FCJP was diluted with sterilized distilled water (10 ml) at room temperature. After approximately 10 min, 1 ml of the dilution was serially diluted 10-fold in NaCl (8.5 g/kg) solution and cultured in agar media. Then the culture plates were incubated at 37°C for 24-48 h and the number of colonies was counted carefully. Chemical compositions of FGLP and FCJP were determined by following the method of AOAC [32]. After preparation of FGLP and FCJP, 0.4% was added with the corn-soybean meal based basal diet. The ingredients and chemical composition of the starter and finisher diet was presented in the Tables 1 and 2. The experimental FGLP and FCJP composition was presented in Table 3. The pH of the FGLP was 3.22–3.29, whereas in FCJP it was 3.35–3.39.

Experimental design, dietary treatments and bird's management

Experimental birds were reared in the Sunchon National University experimental farm, Suncheon, Republic of Korea. A total of 150 one day-old Ross 308 broiler chicks were assigned to five treatment groups having five replications with six birds based on the

Item	Starter diet (0 to 21 days)			Finisher diet (22 to 35 days)		
	Control	FGLP[1]	FGLP[2]	Control	FGLP[1]	FGLP[2]
Ingredients (%, as fed basis)						
Corn grain	57.58	57.18	57.18	60.64	60.24	60.24
Soybean meal	26.80	26.80	26.80	24.90	24.90	24.90
Corn gluten	5.00	5.00	5.00	3.50	3.50	3.50
Soybean oil	2.20	2.20	2.20	2.20	2.20	2.20
Animal fats	4.50	4.50	4.50	5.00	5.00	5.00
Salt	0.25	0.25	0.25	0.25	0.25	0.25
Dicalcium phosphate	2.14	2.14	2.14	2.00	2.00	2.00
Limestone	0.92	0.92	0.92	0.88	0.88	0.88
Vitamin-mineral premix[1]	0.30	0.30	0.30	0.30	0.30	0.30
Choline	0.08	0.08	0.08	0.07	0.07	0.07
L-lysine HCL (78%)	0.24	0.24	0.24	0.16	0.16	0.16
DL-Methionine	0.20	0.20	0.20	0.10	0.10	0.10
Ginkgo probiotics 5%	-	0.40	-	-	0.40	-
Ginkgo probiotics 10%	-	-	0.40	-	-	0.40
Composition (% DM)						
ME (MJ/kg)	13.03	13.01	13.01	13.27	13.25	13.23
Moisture	12.07	11.25	11.02	13.08	13.28	13.25
Crude protein	20.89	20.67	20.98	19.12	18.53	18.11
Ether extract	4.65	4.57	5.93	2.43	3.68	3.33
Crude fiber	4.42	4.45	4.47	3.71	3.00	3.07
Crude ash	5.63	5.14	5.44	5.61	4.99	5.04
Calcium	1.05	1.05	1.05	0.81	0.81	0.81
Available phosphorus	0.55	0.55	0.55	0.45	0.45	0.45
Lysine	1.42	1.42	1.42	1.10	1.10	1.10
Methionine	0.49	0.49	0.49	0.45	0.45	0.45

[1]Vitamin-mineral mixture provided the following nutrients per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 1,500 IU; vitamin E, 20.0 mg; vitamin K3, 0.70 mg; vitamin B12, 0.02 mg; niacin, 22.5 mg; thiamine, 5.0 mg; folic acid, 0.70 mg; pyridoxine, 1.3 mg; riboflavin, 5 mg; pantothenic acid, 25 mg; choline chloride, 175 mg; Mn, 60 mg; Zn, 45 mg; I, 1.25 mg; Se, 0.4 mg; Cu, 10.0 mg; Fe, 72 mg; Co, 2.5 mg (Bayer Korea Ltd., Dongjak-Ku, Seoul, Korea).
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 1: Feed ingredients and chemical composition of experimental diet with fermented *Ginkgo biloba* probiotics.

Item	Starter diet (0 to 21 days)			Finisher diet (22 to 35 days		
	Control	FCJP[1]	FCJP[2]	Control	FCJP[1]	FCJP[2]
Ingredients (%, as fed basis)						
Corn grain	57.58	57.18	57.18	60.64	60.24	60.24
Soybean meal	26.80	26.80	26.80	24.90	24.90	24.90
Corn gluten	5.00	5.00	5.00	3.50	3.50	3.50
Soybean oil	2.20	2.20	2.20	2.20	2.20	2.20
Animal fats	4.50	4.50	4.50	5.00	5.00	5.00
Salt	0.25	0.25	0.25	0.25	0.25	0.25
Dicalcium phosphate	2.14	2.14	2.14	2.00	2.00	2.00
Limestone	0.92	0.92	0.92	0.88	0.88	0.88
Vitamin-mineral premix[1]	0.30	0.30	0.30	0.30	0.30	0.30
Choline	0.08	0.08	0.08	0.07	0.07	0.07
L-lysine HCL (78%)	0.24	0.24	0.24	0.16	0.16	0.16
DL-Methionine	0.20	0.20	0.20	0.10	0.10	0.10
Citron probiotics 5%	-	0.40	-	-	0.40	-
Citron probiotics 10%	-	-	0.40	-	-	0.40
Chemical composition (%DM)						
ME (MJ/kg)	13.03	13.01	13.01	13.27	13.25	13.23
Moisture	12.07	11.84	11.94	13.08	12.71	12.75
Crude protein	20.89	20.67	20.98	19.12	19.22	19.59
Ether extract	4.65	4.05	4.40	2.43	2.50	2.40
Crude fiber	4.42	4.58	4.46	3.71	3.45	3.00
Crude ash	5.63	5.14	5.44	5.61	5.08	5.02
Calcium	1.05	1.05	1.05	0.81	0.81	0.81
Available phosphorus	0.55	0.55	0.55	0.45	0.45	0.45
Lysine	1.42	1.42	1.42	1.10	1.10	1.10
Methionine	0.49	0.49	0.49	0.45	0.45	0.45

[1]Vitamin-mineral mixture provided the following nutrients per kilogram of diet: vitamin A, 15,000 IU; vitamin D3, 1,500 IU; vitamin E, 20.0 mg; vitamin K3, 0.70 mg; vitamin B12, 0.02 mg; niacin, 22.5 mg; thiamine, 5.0 mg; folic acid, 0.70 mg; pyridoxine, 1.3 mg; riboflavin, 5 mg; pantothenic acid, 25 mg; choline chloride, 175 mg; Mn, 60 mg; Zn, 45 mg; I, 1.25 mg; Se, 0.4 mg; Cu, 10.0 mg; Fe, 72 mg; Co, 2.5 mg (Bayer Korea Ltd., Dongjak-Ku, Seoul, Korea).
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 2: Feed ingredients and chemical composition of experimental diet with *Citrus junos* probiotics.

completely randomized design. Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: Corn-soybean meal based basal diet+5% FGLP, 3) FGLP2: Corn-soybean meal based basal diet+10% FGLP, 4) FCJP1: Corn-soybean meal based basal diet+5% FCJP, 5) FCJP2: Corn-soybean meal based basal diet+10% FCJP. The basal diet was formulated to meet the Nutrient Requirements of Poultry (National Research Council, NRC, 1994, Washington DC, USA) and applied for a total of 5 weeks in two stages: Starter (0-3 weeks) and finisher (4-5 weeks). All diets were in mashed form.

To conduct the present experiment, all guidelines for the care and use of animals in research were followed based on the Korean Ministry for Food, Agriculture, Forestry and Fisheries (2008). Broilers were reared in a closed, ventilated, wire-floor caged broiler house (100 cm long × 90 cm wide × 40 cm high/cage) with a floor space of 1,125 cm^2/bird. The cages had a linear feeder in the front and a nipple drinker in the back to provide *ad libitum* feed intake and free access to water. The internal temperature of the broiler house was set and maintained at 34°C for the first week, after which it was gradually reduced to 23°C at 3°C per week, and then maintained at this temperature until the end of the total experimental period. The internal relative humidity was maintained at around 50% throughout the experimental period.

Measurement of growth performance

Continuous lighting was provided for the entire experimental period, and there was no vaccination or medication program. Chicks were inspected daily and dead birds were removed following recoding of the mortality (pen, date and body weight). Feed intake and Body Weight (BW) were recorded weekly by replicate, and the Average Daily Feed Intake (ADFI), Average Daily Gain (ADG), and FCR (feed

Item	Dietary treatments			
	FGLP[1]	FGLP[2]	FCJP[2]	FCJP
Chemical composition				
Moisture (%)	23.42	26.12	30.62	29.80
Crude protein (%)	10.98	15.13	14.84	15.72
Crude fat (%)	2.41	2.07	1.72	1.51
Crude fiber (%)	9.83	9.33	8.14	7.38
Crude Ash (%)	6.66	6.49	5.60	5.52
Nitrogen free extract (%)	53.30	40.86	39.08	40.07
Microbial strains				
Lactobacillus plantarum KCTC 3099	2.0×10^9	2.0×10^9	2.0×10^9	2.0×10^9
Lactobacillus acidophilus KCTC 3111	2.2×10^9	2.2×10^9	2.1×10^9	2.1×10^9
Saccharomyces cerevisiae KCTC 7904	2.4×10^8	2.4×10^8	2.5×10^8	2.5×10^8

Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 3: Chemical composition of fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics.

to gain ratio) per cage were then calculated by period and for the total experimental period.

Collection and analyses of blood and caecal samples

At the termination of the feeding trial, 2 birds close to the mean body weight were randomly selected from each pen for blood sample collection. Blood samples were collected (10 mL) from the wing veins of the selected birds into a 10-mL anticoagulant-free vacutainer tube (Greiner Bio-One GmbH, Kremsmunster, Austria). The samples were subsequently stored on ice during the period of collection and then immediately centrifuged to separate the serum (centrifugation for 15 min at 1,610 × *g* at 4°C). Then, the serum samples were carefully transferred to plastic vials and stored at −20°C until immunoglobulin analysis was performed. The concentrations of serum IgG, IgA, and IgM were assayed using appropriately diluted samples by a sandwich ELISA with chicken-specific IgG (Cat. No. E30–104), IgA (Cat. No. E30–103), and IgM (Cat. No. E10–101) ELISA quantitation kits (Bethyl Laboratories Inc., Montgomery, TX) according to the manufacturer's instructions. Each experiment was run in duplicate and the results represent the means of triplicate experiments. The absorbance of each well at 450 nm was measured within 30 min using a microplate autoreader (Thermo Lab Systems, Helsinki, Finland). The concentrations of IgG, IgA, and IgM were determined using standard curves constructed from the respective immunoglobulin standards and the results were expressed as mg/ml of serum.

Selected chickens were slaughtered at the end of 5th week of experimental period to measure the micro flora concentration of caeca, where caecal contents were collected carefully from each bird. Feed withdrawal period of 12 h were maintained. The collected caecal contents were serially diluted in sterile saline in the 1:10 dilution and then cultured on agar media (duplicate for each). The culture media for *E. coli*, *Salmonella*, *Lactobacillus* and yeast were MacConkey Sorbitol Agar; Salmonella Shigella Agar; Lactobacilli MRS (Mann, Rogosa and Sharpe) Agar; and Potato Dextrose Agar, respectively. Incubation in the anaerobic condition at 37°C for 24 h (*E. coli* and *Salmonella)* and 48 h (*Lactobacillus* and yeast) were done followed by the smearing of supernatant of 100 µl onto the agar plate. Following enumeration of microbial colonies in the duplicate incubated agar plates, microbial counts were expressed as log10 CFU/ml.

Slaughtering and meat sampling procedure

At the end of the experimental period, two broilers from each replicate cage were randomly selected and slaughtered following the halal slaughtering method. The breast and thigh meat were then excised from the carcass by removing the skin, bones and connective tissue. After weighing the breast and thigh meat samples from each bird, they were ground separately using a meat grinder. The samples were subsequently divided into two parts, one for the oxidative stability analysis and another for the fatty acid composition analysis. Finally, the samples were poured into plastic sample bottles, after which those for oxidative rancidity analysis were refrigerated at 4°C and samples for other analyses were stored at -20°C.

Determination of breast and thigh meat fatty acids

The fatty acids compositions of breast and thigh meat were determined by a direct method for Fatty Acid Methyl Ester (FAME) synthesis using a slight modification of the method described by O'Fallon et al. 1 g of minced meat sample was placed into a 15 ml Falcon tube, after which 0.7 ml of 10 N KOH in water and 6.3 ml of methanol were added. The tube was then incubated in a 55°C water bath for 1.5 h with vigorous hand-shaking for 10 s every 30 min to properly permeate, dissolve and hydrolyze the sample. After cooling to below room temperature in a cold tap water bath, 0.58 ml of 24 N H_2SO_4 in water was added. The tube was then mixed by inversion, after which K_2SO_4 precipitated. The sample with the precipitate was incubated again in a 55°C water bath for 1.5 h with vigorous hand-shaking for 10 s every 30 min. After FAME synthesis, the tube was cooled in a cold water bath. Next, 3 ml of hexane were added and the tube was vortexed for 5 min on a multitude vortexed. The tube was subsequently centrifuged for 5 min at 3000 × g (HANIL, Combi-514R, and Korea), after which the top (hexane) layer containing the FAME was dehydrated through the anhydrous Na_2SO_4. The extracted and dehydrated hexane was then concentrated to 1.5 ml and placed into a GC vial for analysis.

The fatty acid composition of the FAME was determined using a Gas Chromatograph (Agilent, 7890A series, USA) equipped with a flame ionization detector and a Hewlett Packard HP-88 capillary column (J&W Scientific, USA) with a length of 60 m, a 0.52 mm internal diameter and a 0.20 µm polyethylene glycol-film thickness. Samples were injected using an auto-sampler (Agilent Technologies 7693, USA). The initial oven temperature was 125°C, which was held for 1 min, then increased to 145°C at 10°C/min, where it was held for 26 min, then further increased to 220°C at 2°C/min, where it was held for 2 min. Purified air and hydrogen were applied at a flow rate

of 400 ml/min and 40 ml/min as the carrier gas, whereas helium was applied at 40 ml/min as the makeup gas. Both the injector and detector temperature were set at 260°C, and the split ratio was 30:1. Fatty acids were identified by comparison of their retention times with those of a standard FAME mixture (Supelco™ 37 Component FAME Mix, 10 mg/ml in CH_2Cl_2. Catalog Number 47885-U. Supelco, Bellefonte, PA 16823-0048, USA). Sums and ratios useful for evaluating the nutritional value and healthiness of the fatty acid profile were also determined; specifically, the sum of saturated fatty acids (ΣSFA), monounsaturated fatty acids (ΣMUFA), polyunsaturated fatty acids (ΣPUFA), n-3 fatty acids (Σn-3), n-6 fatty acids (Σn-6) and the ratios of MUFA to SFA (MUFA/SFA), PUFA to SFA (PUFA/SFA), n-6 to n-3 (n-3/n-6) and hypocholesterolemic to hypocholesterolemic (H/H) fatty acid ratio. The H/H ratio was determined as follows:

H/H=[(sum of C18:1 cis-9, C18:2 n-6, C20:4n-6, C18:3 n-3, C20:3n-6, C20:5 n-3, and C22:6 n-3)/(sum of C14:0 and C16:0)].

Statistical analyses

All data were subjected to ANOVA using the General Linear Models (GLM) function of the Statistical Analysis System (SAS, 2003, Version 9.1, SAS Institute, Cary, NC, USA). Each cage was considered as the experimental unit for growth performance parameters (BW, BWG, FI and FCR), whereas an individual bird served as the experimental unit for immunity and caecal microbiology. A probability level of P<0.05 was considered as statistically significant and a level of P<0.10 was considered as statistical tendency.

Results

Performance of broilers

The result of the growth performance of the broilers supplemented with fermented ginkgo leaf probiotics and fermented citron rind probiotics was presented in Table 4. The result depicted that, the body weight gain and feed intake of broilers were significantly improved after supplementation of FGLP2 during starter, finisher and overall period while compared with the control diet and FCJP group (P<0.05). Feed conversion efficiency (FCR) did not differ during starter and finisher period, however, it was found better in FCJP2 in comparison to the control group during overall period of experiment (P<0.05). During finisher period, FCJP2 differed with FGLP1 and FCJP1 (P<0.05), while during overall period, FCJP differed with FGLP1, FGLP2 and FCJP1 (P<0.05).

Mortality and immunity of birds

The mortality rate of the birds as 5.56% in control group and 4.5% FGLP; while FCJP2 showed the zero mortality (data not shown). The serum immunoglobulin status is shown in Table 4. The serum immunoglobulin G (IgG) levels were significantly elevated in the FGLP and FCJP supplemented groups relative to control group (P<0.05). Where, serum IgM level differed in FCJP2 with FGLP1, FGLP2 and FCJP1 group (P<0.05).

Cecal microbiology and pH

The cecal microbiology data elucidated that, there was no significant differences among the dietary treatments of FGLP and FCJP (Table 5). The pH of the cecal content did not differ after dietary inoculation of FGLP and FCJP in the broiler diet (P>0.05).

Chemical composition and cholesterol content of breast and thigh meat

Table 6 shows the meat composition of breast and thigh portion, where it was depicted that, breast and thigh meat crude protein and crude fat content was affected after dietary inclusion of FGLP and FCJP

Items	Dietary treatments					SEM	P value
	Control	FGLP[1]	FGLP[2]	FCJP[1]	FCJP[2]		
0-3 weeks							
Initial weight(g)	39	39	39	39	39	0.02	0.21
Final weight(g)	764^{c}	759^{c}	855^{a}	798^{b}	763^{c}	7.41	<0.0001
Weight gain(g)	724^{c}	720^{c}	816^{a}	759^{b}	724^{c}	7.41	<0.0001
Feed intake(g)	1,013^{b}	1,027^{b}	1,167^{a}	1,025^{b}	998^{b}	19.18	0.001
FCR (Feed/Gain)	1.40	1.43	1.43	1.35	1.38	0.02	0.27
3-5 weeks							
Initial weight(g)	764^{c}	759^{c}	855^{a}	798^{b}	763^{c}	7.41	<0.0001
Final weight(g)	1,870^{c}	1,890^{c}	2,097^{a}	1,895^{c}	1,964^{b}	15.40	0.001
Weight gain(g)	1,106^{bc}	1,130^{bc}	1,242^{a}	1,097^{c}	1,201^{ab}	20.07	0.02
Feed intake(g)	1,786^{c}	1,896^{ab}	1,977^{a}	1,818^{bc}	1,860^{bc}	26.75	0.01
FCR (Feed/Gain)	1.61^{ab}	1.68^{a}	1.60^{ab}	1.66^{a}	1.55^{b}	0.03	0.08
0-5 weeks							
Initial weight(g)	39	39	39	39	39	0.02	0.21
Final weight(g)	1,870^{c}	1,890^{c}	2,097^{a}	1,895^{c}	1,964^{b}	10.89	<0.0001
Weight gain(g)	1,830^{c}	1,850^{c}	2,058^{a}	1,855^{c}	1,925^{b}	10.88	<0.0001
Feed intake(g)	2,800^{d}	2,924^{b}	3,144^{a}	2,844^{cd}	2,858^{c}	12.15	<0.0001
FCR (Feed/Gain)	1.53^{b}	1.58^{a}	1.53^{b}	1.53^{b}	1.48^{c}	0.01	0.001

$^{a-d}$Mean with different superscripts within the same row are significantly different (P<0.05).
SEM: Standard Error of Mean.
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 4: Effects of dietary fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics on growth performance of broilers.

Item	Dietary treatments					SEM	P value
	Control	FGLP[1]	FGLP[2]	FCJP[1]	FCJP[2]		
Immunoglobulins (mg/mL)							
Immunoglobulin M (IgM)	0.21[a]	0.16[b]	0.14[b]	0.14[b]	0.25[a]	0.01	0.01
Immunoglobulin A (IgA)	0.51	0.52	0.58	0.61	0.54	0.03	0.17
Immunoglobulin G (IgG)	0.23[b]	0.29[ab]	0.32[a]	0.32[a]	0.33[a]	0.01	0.04
Cecum microbiota (log10 cfu/g)							
Lactic acid bacteria	7.33	6.79	7.60	7.39	8.01	0.35	0.27
Yeast & mold	7.36	6.90	7.41	7.22	7.62	0.29	0.61
Escherichia coli	6.49	4.73	7.64	6.59	8.55	0.53	0.20
pH	5.85	6.12	6.20	5.72	5.59	0.30	0.62

[a,b]Mean with different superscripts within the same row are significantly different (P<0.05).
SEM: Standard Error of Mean.
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 5: Effects of dietary fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics on serum immunoglobulin concentration and cecal microbiology in broilers.

Meat composition	Dietary treatments					SEM	P value
	Control	FGLP[1]	FGLP[2]	FCJP[1]	FCJP[2]		
Breast meat							
Crude protein (%)	24.95[a]	23.52[ab]	22.80[b]	23.71[ab]	22.22[b]	0.46	0.01
Crude fat (%)	0.95[d]	1.05[cd]	2.04[a]	1.22[bc]	1.43[b]	0.08	<0.0001
Moisture (%)	74.35[ab]	74.62[a]	74.17[ab]	73.85[b]	74.60[a]	0.21	0.10
Crude ash (%)	1.36[a]	1.29[ab]	1.21[b]	1.40[a]	1.29[ab]	0.03	0.01
Thigh meat							
Crude protein (%)	19.03[c]	19.34[bc]	19.70[ab]	20.70[a]	20.45[ab]	0.39	0.03
Crude fat (%)	2.00[b]	2.90[a]	3.37a	3.53[a]	3.01[a]	0.28	0.01
Moisture (%)	75.35	74.34	74.51	74.43	75.12	0.37	0.27
Crude ash (%)	1.08	1.18	1.09	1.21	1.12	0.03	0.16
Cholesterol (mg/100 g)							
Breast meat	140.89[ab]	156.99[a]	147.66[ab]	120.99[b]	122.16[b]	5.53	0.01
Thigh meat	165.39[ab]	183.27[a]	146.14[ab]	162.30[ab]	133.41[b]	8.85	0.03

[a,b]Mean with different superscripts within the same row are significantly different (P<0.05).
SEM: Standard Error of Mean.
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 6: Effects of dietary fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics on breast and thigh meat composition in broilers.

in the broiler diet. In case of breast meat the crude protein content was decreased whereas the crude fat content was increased in FGLP2 and FCJP2 relative to control group (P<0.05). Moisture content did not differ with control group; but there was found higher moisture content in the FGLP1 and FCJP2 group compared to FCJP1 group (P<0.05). The crude ash content was lower in FGLP2 relative to the control group. Where in case of thigh meat both the crude protein and crude fat content was augmented in FGLP and FCJP supplemented group compared to control (P<0.05). The meat cholesterol content did not differ in FGLP and FCJP while compared with the control group; however, it did differ between the FGLP and FCJP group (P<0.05).

Fatty acid composition of breast and thigh meat

The fatty acid composition of breast and thigh portion of broiler carcass was presented in the Tables 7 and 8. In Table 7, for the breast meat, it was observed that, sum of Saturated Fatty Acid (SFA) and sum of Polyunsaturated Fatty Acid (PUFA) did not differ in FGLP and FCJP in comparison to the control; however, the sum of Monounsaturated Fatty Acid (MUFA) was lower in FGLP and FCJP group relative to the control group (P<0.05). The sum of n-3 fatty acid was observed higher in FGLP1 compared to control whereas the sum of n-6 fatty acid was found lower in FGLP1 in comparison to the control group (P<0.05). There was found no significant differences in the ratio of MUFA to SFA and PUFA to SFA; however, the ratio of n-6 PUFA to n-3 PUFA was differed in FGLP1, FCJP1 and FCJP2 compared to the control group (P<0.05).

As shown in Table 8, in case of thigh meat, the sum of SFA was downtrended in the FGLP1 and FGLP2 group relative to the control group (P<0.05). The sum of MUFA and PUFA did not show any difference due to supplementation of FGLP and FCJP. The sum of n-3 PUFA did show difference among the control, FGLP and FCJP group. While the sum of n-6 PUFA significantly differed in the FGLP1 in relation to the control group (P<0.05).

Discussion

Medicinal plants or their materials contain several types of bioactive compounds. The chemical constituents of the *Ginkgo biloba* leaf are polysaccharides, flavonoids, flavone glycosides, quercetin, terpenoids, bilobalides and ginkolides [33,34]. The *Citrus junos* composed of flavonoids, naringin, hesperidin, carotinoids, polyphenols, and

Fatty acid (g/100 g FA)	Dietary treatments					SEM	P value
	Control	FGLP[1]	FGLP[2]	FCJP[1]	FCJP[2]		
Myristic acid (C14:0)	0.93[b]	1.65[ab]	1.60[ab]	2.00[a]	2.14[a]	0.17	0.04
Pentadecanoic acid (C15:0)	2.24[a]	2.28[a]	1.09[b]	0.96[b]	0.97[b]	0.13	<0.0001
Palmitic acid (C16:0)	21.11	20.45	19.92	20.23	19.96	0.34	0.27
Palmitoleic acid (C16:1n7)	4.38	4.15	4.39	4.03	3.76	0.23	0.39
Stearic acid (C18:0)	0.21[b]	5.50[a]	9.71[a]	7.36[a]	7.01[a]	1.35	0.01
Oleic acid (C18:1 n9)	35.08[a]	32.95[b]	36.16[a]	34.73[a]	34.73[a]	0.38	0.001
Linoleic acid (C18:2n6)	17.85[a]	15.25[c]	17.37[a]	16.08[b]	17.33[a]	0.26	0.002
α-linolenic acid (C18:3n3)	0.43[a]	0.34[b]	0.45[a]	0.47[a]	0.44[a]	0.02	0.06
Arachidic acid (C20:0)	0.74[a]	0.48[b]	0.77[a]	0.64[a]	0.71[a]	0.04	0
Eicosaenoic acid (C20:1n9)	0.5	0.45	0.45	0.55	0.59	0.05	0.29
Eicosapentanoic acid (C20:5n3)	4.19[b]	5.70[a]	3.23[b]	4.28[b]	4.27[b]	0.34	0.01
DGLA (C20:3n6)	1.99	1.72	1.75	2.11	2.01	0.14	0.43
Docosahexaenoic acid (C22:6n3)	1.12[b]	2.78[a]	1.31[b]	1.94[ab]	1.61[b]	0.25	0.02
Tetracosaenoic acid (C24:1n9)	1.40[ab]	1.54[ab]	1.10[b]	1.66[ab]	1.91[a]	0.16	0.05
∑SFA	34.01	35.35	33.56	34.52	34.11	0.68	0.59
∑MUFA	41.35[a]	44.08[b]	42.09[b]	40.96[b]	40.99[b]	0.53	0.04
∑PUFA	25.57	25.78	24.1	24.88	25.65	0.51	0.21
∑n-3	5.74[bc]	8.81[a]	4.98[c]	6.69[b]	6.31[b]	0.37	<0.0001
∑n-6	19.84[ab]	16.96[c]	19.11[a]	18.20[b]	19.34[ab]	0.4	0.001
MUFA/SFA	1.22	1.12	1.25	1.19	1.21	0.03	0.17
PUFA/SFA	0.75	0.74	0.72	0.72	0.75	0.02	0.77
n-6/n-3	3.53[ab]	1.97[d]	3.87[a]	2.77[c]	3.12[bc]	0.18	<0.0001

[a,b]Mean with different superscripts within the same row are significantly different (P<0.05).
SEM: Standard Error of Mean.
∑SFA: Saturated fatty acid; ∑MUFA: Mono-unsaturated fatty acid; ∑PUFA: Poly-unsaturated fatty acid, ∑n-3: Total omega 3 fatty acid; ∑n-6: Total omega 6 fatty acid.
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 7: Effects of dietary fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics on the fatty acid composition of broiler thigh meat.

Fatty acid (g/100g FA)	Dietary treatments					SEM	P value
	Control	FGLP[1]	FGLP[2]	FCJP[1]	FCJP[2]		
Myristic acid (C14:0)	1.1	1.03	1.07	1.02	1.07	0.02	0.16
Myristoleic acid (C14:1 n5)	0.38[a]	0.37[ab]	0.34[b]	0.35[b]	0.34[b]	0.01	0.03
Pentadecanoic (C15:0)	0.79	0.83	0.71	0.81	0.85	0.07	0.72
Palmitic acid (C16:0)	21.54[a]	20.84[b]	20.06[c]	21.18[ab]	20.86[b]	0.17	0.001
Palmitoleic acid (C16:1n7)	6.14[ab]	6.75[a]	5.52[b]	5.84[b]	5.57[b]	0.23	0.01
Stearic acid (C18:0)	7	6.66	7.42	7.12	7.31	0.21	0.13
Oleic acid (C18:1 n9)	39.72	40.35	39.94	40.18	40.06	0.45	0.92
Linoleic acid (C18:2n6)	17.85[ab]	16.70[c]	18.22[a]	17.21[bc]	18.22[a]	0.26	0.001
α-linolenic acid (C18:3n3)	0.57	0.58	0.61	0.61	0.58	0.02	0.43
Arachidic acid (C20:0)	1.03[ab]	0.94[c]	0.99[bc]	0.99[bc]	1.06[a]	0.02	0.01
Eicosaenoic acid (C20:1n9)	0.21[b]	0.21[b]	0.25[a]	0.23[ab]	0.24[b]	0.01	0.01
Eicosadienoic acid (C20:2 n6)	0.14	0.15	0.15	0.16	0.14	0.01	0.33
DGLA(C20:3n6)	0.37	0.4	0.37	0.46	0.41	0.04	0.59
Eicosapentanoic acid (C20:5n3)	2.15[b]	3.07[a]	2.19[b]	2.70[ab]	2.23[b]	0.36	0.04
Docosahexaenoic acid (C22:6n3)	0.88	0.52	0.36	0.99	0.83	0.19	0.16
∑SFA	31.46[a]	30.30[b]	30.25[b]	31.10[a]	31.15a	0.24	0.001
∑MUFA	46.44	47.68	46.05	46.6	46.21	0.61	0.43
∑PUFA	21.95	21.43	21.89	22.11	22.41	0.6	0.86
∑n-3	3.61[b]	4.17[a]	3.16[b]	4.28[a]	3.64[b]	0.48	0.05
∑n-6	18.35[ab]	17.25[c]	18.73[a]	17.83[bc]	18.76[a]	0.25	0.001
USFA/SFA	1.48	1.57	1.52	1.5	1.49	0.03	0.15
PUFA/SFA	0.7	0.71	0.73	0.71	0.72	0.02	0.86
n-6/n-3	5.44	4.5	2.77	4.44	5.18	1.46	0.11

[a-c]Mean with different superscripts within the same row are significantly different (P<0.05).
SEM: Standard Error of Mean.
∑SFA: Saturated fatty acid; ∑MUFA: Mono-unsaturated fatty acid; ∑PUFA: Poly-unsaturated fatty acid; ∑n-3: Total omega 3 fatty acid; ∑n-6: Total omega 6 fatty acid.
Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: 5% FGLP (corn-soybean meal based basal diet+5% FGLP), 3) FGLP2: 10% FGLP (corn-soybean meal based basal diet+10% FGLP), 4) FCJP1: 5% FCJP (corn-soybean meal based basal diet+5% FCJP), 5) FCJP2: 10% FCJP (corn-soybean meal based basal diet+10% FCJP).

Table 8: Effects of dietary fermented *Ginkgo biloba* probiotics and *Citrus junos* probiotics on the fatty acid composition of broiler thigh meat.

flavonols, pectins, acids, volatile oils, enzymes and vitamin C [35,36]. The active phytochemicals present in *Punica granatum* are punicalagin, flavonoids, peduculagin, ellagitannin, and punicalin, some other phenolic compounds and anthocyanins [36]. Fermentation of plant materials with the beneficial microbe's results improvement of the fermented product with the enrichment of the enzymes, vitamins and growth factors which can helps to promote the performance of animals [37,38]. The combination of probiotics and prebiotics that include both beneficial microorganisms and substrates exerts synergistic effects on the gastro-intestinal tract and consequently promotes the growth of animals [39]. Ginkgo biloba while fermented with *Aspergillus* spp. reported that there was no impact on weight gain in broilers [40]. However, in the present study, the weight gain was improved in the FGLP2 group compared to control and FCJP group. Supporting to the study, it was reported that, supplementation of fermented *Ginkgo biloba* along with multi-microbe probiotic was effective in the improvement of the growth performance of broilers [26].

Fermentation of *Citrus junos* with *Saccharomyces cerevisiae* or *Bacillsu subtilis* can improve the nutritive value and antioxidative value in the fermented product resulting in the improvement of the weight gain and feed efficiency [41,42]. No improvement in the feed efficiency after supplementation of citrus fruits by-products with fermentation of multi-microbe probiotics was reported in the study of Ahmed et al. [31]. However, in the present study, although the weight was not significantly improved, the feed conversation efficiency was improved in the FCJP group compared to control. The pectin content of *Citrus junos* might be influenced by the probiotic organisms and activate to generate pectinolytic enzymes resulting in higher production of lactic acid bacteria [36,43,44]; where lactic acid bacteria can complement with the other beneficial microbes can exhibit synergism to utilize the feed with higher efficiency in broiler [45]. Therefore, combination of flavonoids, naringin, hesperidin, carotinoids, polyphenols, and flavonols, pectins, punicalagin, flavonoids, peduculagin, ellagitannin, and punicalin, some other phenolic compounds with the microbial generated organic compounds [34-36], the feed efficiency was improved in the FCJP2 group. The feed efficiency data depicted that, lower level of both the FGLP and FCJP was more efficient in case of broiler. It was reported that lower percentage of fermented *Citrus junos* or *Alisma canaliculatum* along with multimicrobe probiotic might be able to improve the performance of broiler but higher percentage is not able to promote the growth performance of broilers [23,31]. Studies of the modes of action of combined phytobiotic substances and probiotics suggested the possibility of synergistic effects between these classes of compounds, which might help improve microbial balance, immune development and animal performance as well [23]. In addition, polysaccharides of *Ginkgo biloba extract* and *Citrus junos* with *Punica granatum* along with probiotics might play synnergysm for the improvement of performance and immunity in birds. The combination of benficial microbes are able to improve the performance of broilers [17,24].

The improved growth enhancement was concurrent with the lower mortality rate and elevation of Immunoglobulin G (IgG) in the FGLP and FCJP supplemented birds in the present study. Plant derived flavones and terpenes from the combination of natural plants along with probiotic ferementation might have immune promoting activites that help enhance the lymphocyte synthesis, phagocytosis activity and cytokin release [46]. Supporting these findings, improvement in the immune status and lower mortality was reported after inclusion of *Ginkgo biloba extract* and *Camelia sinensis* in broilers. The elevation serum immnonoglobulin level indicating the natural antibodies impact which might be attributable to enlargement of the splenic lymphocite due to the probiotic effect and combination of polyphenolic compounds (derived from *Punica granatum*, *Ginkgo biloba* and *Citrus junos*) [47]. Supplementation of probiotics elevate the capability of the splenic lymphocytes to the proliferation in tesponse to the B-cell and T-cell mitogenic stimulation [48,49]. Dietary inclusion of 10% fermented *Gingkgo biloba* reported to be beneficial to improve immune function in the case of weaned piglets [50], which in turn supports the results of our study regarding higher serum immunoglobulin levels in the case of FGLP supplementation of broilers. The presence of flavonoids (dominance of hesperidin) and vitamin C (L-ascorbic acid) in case of *Citrus junos* and fermentation with probiotics ascribed to be the reason of improvement in the humoral immunity of birds [47,51].

Hossain et al. [23] reported that, the composition of meat can be affected by *Alisma canaliculatum* with probiotics, where they reported that, crude protein was increased but crude fat is decreased. Similar trend was reported by Bostami et al. [26] while they tested the efficacy of Fermented *Salicornia herbacia* L. and *Houttuynia cordata* Thunb with multi-microbe probiotics in broilers. However, in the present study elevation of crude protein and diminishing of crude fat was exhibited in the FGLP2 and FCJP2 in case of breast meat; where for the thigh meat both crude protein and crude fat was increased in FGLP and FCJP compared to control. The reason of such types of result for the thigh meat is not clear to us. Currently, due to health aspects, peoples are paying attention to the new natural products having lipid reducing activity, so that cholesterol level can be controlled and checked the hyperlipidemic risk factor [52-54]. Plant derived secondary metabolites can play important role on the cholesterol content through the mode of action in the biological reactions. The plant bioactive compounds exhibited anti-oxidative action, anti-tumor and anti-lipidemic action (lowering the cholesterol level [40,55]. The biologically active substances can play in favor of diminishing the cholesterol level [48,56]. It was reported that, plant metabolites influences on abate mating the serum level of TC and LDL-C [56]. The plant bioactive compounds might be able to format an insoluble complex combining with the endogenous cholesterol which can further prevented to reabsorb by the action of bile in the physiological system [57,58]. Addition of probiotic in the diet of broiler reported to be effective on reducing the serum cholesterol, low density lipoprotein and triglycerides [59]. Therefore, in the present study, the physiological reaction of the combination of plant materials along with multi-microbes in the FCJP group might effectively exhibited on reducing the breast and thigh meat cholesterol content, which indicated the better quality of broiler meat. Cardiovascular disease is a common worldwide and major case of health which usually happened due to the cholesterol level [60]. Increment of the Low Density Lipoprotein (LDL) causes acceleration of atherosclerosis and finally coma and death, therefore limiting cholesterol intake might be the effective approach [40,55].

Fatty acids play major role in the human nutrition and also have different and important functions in plant, mammal, and animal metabolism. Fatty acid acts as precursor of the biosynthesis of eicosanoids which is considered as an important bio-regulator in the body physiology and cellular metabolic processes. Fatty acids profile in food has a direct impact on human health according to the epidemiological study [61,62]. It well known fact that, the unsaturated fats have a hypocholesterolemic effect, whereas the saturated fats tend to proliferate the level of the total cholesterol and Low-Density Lipoproteins (LDL) [63]. The diminishing of the unsaturated fatty acids will resulted on the compositional change in the membrane lipids which consequently will impact on the membrane lipid phase

transition [64]. Ahmed et al. [24] reported that, plant secondary metabolites of *Punica granatum* able to reduce the total SFA with different dose level in broiler breast and thigh meat. Bostami et al. [26] reported that supplementation of medicinal plants (*Salicornia herbacea* and *Houttuynia cordata*) and multi-microbe probiotics is potential to ameliorate the fatty acids due to the presence of plant phenolic compounds and organic acids of fermented product. In the present study, for breast meat there was found no impact on sum of SFA and sum of PUFA, but in case of thigh meat sum of SFA was diminuted in the FGLP group relative to control and FCJP group, which is indicated the positive manner on the aspect of meat quality and health risk.

Usually the variation in the SFA, MUFA and PUFA is happened due to the phenomena of conversion of one fatty acid to another, like stearic to oleic acid; and the action of enzyme (such as fatty acid synthase enzyme) in the formation and depletion of fatty acids [65]. The sum of MUFA was declined in the FGLP and FCJP group in the present study. The reduction of SFA and MUFA could be happed due to the action of plant derived flavonoids, tannins, mixture of polyphenolics in goat, sheep and broiler meat [66,67]. The key enzyme associated with the conversion and elevation-diminution process of fatty acid is the 9-desaturase enzyme [68]. It was postulated that, the flavonoids, polyphenols and organic acids from the FGLP and FCJP might influence on the function of 9-desaturase enzyme, which consequently resulted in the declining of the MUFA in the present study. The sum of n-3 PUFA was increased but n-6 PUFA was decreased in FGLP1 (both in breast and thigh meat) and in FCJP1 (in thigh meat) in the current study. The phenomena regarding the enlargement and abatement of the n-3 and n-6 PUFA in the present study might be attributable to the competition for the similar enzymes during elongation and desaturation metabolism of fatty acids in the physiological system [69]; the metabolites derived from the combination of plant materials along with multimicrobe probiotics in the FGLP and FCJP might affect the enzymatic system which influence in the composition of fatty acids in the present study. Consumers are now more interested in consuming the product having rich amount of n-3 PUFA to combat against the cardiovascular disease; therefore, Eicosapentaenoic Acid (EPA) and Docosahexaenoic (DHA) acids are two most important n-3 fatty acids for the human nutrition. EPA and DHA acts as the precursor of prostaglandin, thromoboxane, leukotriene eicosanoids and resolvins which play significant role against heat attack, stroke, and anti-inflammatory function [70,71]. DHA acts as the precursor of the protectins and resolvins which acts as anti-inflammatory and neuroprotective agent [70,72]; and also important for the functional development of the brain of young and for the maintenance of the brain of adult human being. In the present study, EPA and DHA (in breast meat) and EPA (in thigh meat) were proliferated in the FGLP1 supplemented group which is the excellent outcome of this research. The variation in the fatty acid and cholesterol content in the present study might be attributable to the variation in the composition of the chemical constituents and also the dose of bioflavonoids derived from the FGLP and FCJP [68].

Conclusion

Present study was conducted to observe the dietary effect of fermented *Ginkgo biloba* probiotics (FGLP) and Fermented *Citrus junos* Probiotics (FCJP) on growth performance, immunity, cecal microbiology, meat composition and fatty acid profile in broilers. Dietary treatments were: 1) Control (corn-soybean meal based basal diet), 2) FGLP1: Corn-soybean meal based basal diet+5% FGLP, 3) FGLP2: Corn-soybean meal based basal diet+10% FGLP, 4) FCJP1: Corn-soybean meal based basal diet+5% FCJP, and 5) FCJP2: Corn-soybean meal based basal diet+10% FCJP. A significant increase in weight gain during starter (0-21 days), finisher (22-35 days) and overall period (0-35 days); and feed intake during finisher period (22-35 days) and overall period (0-35 days) was exhibited in the FGLP2 supplemented group compared to control and other treatment groups. Better feed conversion efficiency was found after FCJP2 supplementation during finisher and overall period compared to control and other treatment groups. Serum IgG was elevated in FGLP and FCJP supplemented group relative to control birds ($P<0.05$). Meat composition data elucidated that, thigh meat crude protein and crude fat content was increased in FCJP1 supplemented group compared to control. There was found no significant differences in meat cholesterol content between control and treatment groups; however, among meat fatty acids, sum of SFA in thigh meat was diminished in FGLP group and sum of MUFA in breast meat was diminuted in the FGLP and FCJP group compared to control. Sum of n-3 PUFA of breast and thigh meat was elevated after supplementation of FGLP and FCJP compared to control. To sum up, dietary FGLP and FCJP supplementation significantly improved performance and immunity, decreased SFA and elevated n-3 PUFA of broiler meat. Therefore, FGLP and FCJP probiotics could be supplemented as functional feed additives in broilers diet. However, further detail research is required to confirm the dose level.

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Polyphenol-rich Blackcurrant Pomace Counteracts Impaired Antioxidant Status and Serum Lipid Profile in Rabbits Fed a Diet High in Unsaturated Fat

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Abstract

Blackcurrant pomace, a by-product of juice fabrication with promising health benefits, is currently an unutilized nutritional resource. In the presented study an influence of dietary supplementation with blackcurrant pomace, constituting a source of antioxidant phytochemicals and fiber, on selected metabolic biomarkers of rabbits was evaluated. A 28-d experiment carried out on 34-d old rabbits addressed the analysis of physiological properties of two types of diet, standard chow and with additional 10% of lard, each supplemented with unprocessed blackcurrant pomace rich in polyphenols or a processed one, partly deprived of polyphenolic fraction. Twenty rabbits were allocated to four groups fed the following diets: Standard chow +15% of either unprocessed or processed blackcurrant pomace or lard-enriched diet +15% of either unprocessed or processed blackcurrant pomace. Irrespectively of the diet type, inclusion of unprocessed, compared to processed blackcurrant pomace, significantly improved antioxidant status of rabbits expressed by lower level of substances reacting with thiobarbituric acid (TBARS) in the liver and kidneys, as well as higher level of serum total antioxidant status (TAS), integral antioxidant capacities of hydrophilic (ACW) and lipophilic (ACL) substances. Upon lard-enriched diet, unprocessed blackcurrant abundant in polyphenols affected more beneficially serum triacylglycerides (TAG), total cholesterol (TC) and insulin levels. The antioxidant, hypolipidemic and hypoinsulinemic action of the blackcurrant supplement should be ascribed to the polyphenolic constituents present in the pomace.

Keywords: Berries; High-fat diet; Oxidative stress; Atherogenic dyslipidemia

Abbreviations: ACL: Integral Antioxidant Capacity of Lipophilic Substances; ACW: Integral Antioxidant Capacity of Hydrophilic Substances; AI: Atherogenic Index; ANOVA: Analysis of Variance; AOAC: Association of Official Analytic Chemists; BL: Processed Blackcurrant Pomace; BR: Unprocessed Blackcurrant Pomace; BW: Body Weight; CP: Crude Protein; DM: Dry Matter; HFBR: Lard-enriched Diet Supplemented with Unprocessed Blackcurrant Pomace; ELISA: Enzyme-linked Immunosorbent Assay; HFBL: Lard-enriched Diet Supplemented with Processed Blackcurrant Pomace; FFA: Free Fatty Acids; GIP: Gastric Inhibitory Polypeptide; GL: Glucose; HDL-C: HDL Cholesterol; HFD: High-fat Diet; HOMA-IR: Homeostasis Model Assessment for Insulin Resistance; HOMA-β: Homeostasis Model Assessment for Pancreatic Insulin Secretion; HPLC: High-performance Liquid Chromatography; IDF: Insoluble Dietary Fiber; LDL-C: LDL-cholesterol; NS: Not Statistically Significant; SBL: Standard Chow Supplemented with Processed Blackcurrant Pomace; SBR: Standard Chow Supplemented with Unprocessed Blackcurrant Pomace; SD: Standard Deviation; SDF: Soluble Dietary Fiber; TAG: Triacylglyceride; TAS: Total Antioxidant Status; TBARS: Substances Reacting with Thiobarbituric Acid; TC: Total Cholesterol; TDF: Total Dietary Fiber

Introduction

Many authors have proposed that the beneficial activity of polyphenolic compounds present in unrefined diet is linked, to a great extent, with physiological effects of dietary fiber constituting the fiber-polyphenols complexes [1,2]. Some of them have even postulated the necessity of characterizing parameters of the antioxidant status of the body at dietary supplementation with different dietary fibers [2]. Esposito et al. [3] conclude results of their study with a statement that the fiber-polyphenols complex seems to be more beneficial to the consumer's health than the highly-purified medicinal preparation of dietary fiber or antioxidants. The fiber-antioxidants complex seems to be a natural route of delivering components with antioxidative properties to colonic microbiota, thus protecting antioxidants against degradation in the stomach [3,4]. These studies point to potential advantages of diet supplementation with natural preparations containing both functional polysaccharides and polyphenolic compounds, which enables utilizing the physiological properties of both groups of these compounds locally in the gut as well as in internal tissues. Our study showed that the addition of the polyphenolic fraction from chicory root to diets containing prebiotic fructans did not diminish the positive effect of inulin and oligofructose on the ecosystem of the gastrointestinal tract, and triggered positive changes in the blood lipid profile as well as deceleration of pro-oxidative processes in selected tissues [5].

Although the technology to obtain blackcurrant juices is highly efficient, from 12% to 25% of processed raw material becomes a by-product and after production is treated as waste mass [6]. The most frequently applied ways of recycling fruit residues are for animal feed and composting or by disposal in dumps; the latter being a

great waste of beneficial compounds present in the pomace (i.e., fiber, polyphenols), where otherwise these could provide a source of components in health food additives thereby bringing economic profits. Several studies indicate the possibility of using extracts from blackcurrant to positively modulate key markers of the health status of consumer's body [7,8], thus pointing at polyphenols as the most active fraction of fruit pomaces [9,10].

In the present study, it was hypothesized that removing a large part of phenolic fraction from blackcurrant pomace through ethanolic extraction would negatively affect parameters describing the lipoprotein profile and antioxidant status of the host. To verify this statement, two types of rabbits' diet, standard chow and with dietary addition of 10% lard, were supplemented with either natural unprocessed (BR) or processed blackcurrant pomace (BL), deprived of most of polyphenols through extraction.

Methods

Blackcurrant pomaces

Commercial blackcurrant pomace was obtained from the manufacture producing concentrated fruit juice (the ALPEX Company, Łęczeszyce, Poland). Fresh pomace from the Bücher type press was dried in a convection oven at a temperature ≤ 70°C until the moisture content was lower than 5%, and then the dried material was passed through sieves with a mesh of 2 mm. The seedless preparation thus obtained constituted the natural, unprocessed blackcurrant pomace. The processed blackcurrant pomace was subsequently prepared when phenolic compounds were partly removed from pomace using extraction with 45% (v/v) ethanol. This extraction was carried out in a column extractor with contact time. The residue obtained after removal of the ethanol and drying in the air oven constituted the processed blackcurrant pomace.

Chemical analyses of the plant material

The chemical composition of both blackcurrant pomaces are detailed in Table 1. Each pomace was analyzed in duplicate for dry matter (DM), crude protein (CP), fat, ash, total dietary fiber (TDF), and insoluble dietary fiber (IDF) using AOAC methods 934.01, 920.152, 930.09, 940.26, 985.29, and 993.19, respectively. Soluble dietary fiber (SDF) was calculated as the difference between TDF and IDF. High-performance liquid chromatography (HPLC) analysis of phenolic compounds in unprocessed and processed blackcurrant pomaces was also performed. Polyphenol compounds were extracted by a mixture solution of the composition of methanol:water:formic acid at a volumetric ratio of 50:48:2 v/v/v. Before analysis, the extracts were centrifuged at 4,800 × g for 5 min. Anthocyanins and other phenolics were analyzed using KNAUER Smartline chromatograph (Berlin, Germany) equipped with two pumps. The compounds in the phenolic extracts were separated on a 150 mm × 4.6 mm i.d., 5 µm Gemini 5u C18 110A column (Phenomenex Synergi, Torrance, CA, USA) using gradient elution with 10% v/v formic acid in water (A) and 50:40:10 v/v/v acetonitrile:water:formic acid (B). The column temperature was set at 40°C. The flow rate was 1 ml/min and the gradient program was as follows: 0-0.6 min, 12% B; 0.6-16 min, 12-30% B; 16-20.5 min, 30-100% B; 20.5-22 min, 100% B; 22-25 min, 100-12% B, 25-35 min, 12% B. The injection volume was 20 µl. The data was collected by the EuroChrom 2000 program (Knauer GmbH, Berlin, Germany). Quercetin and myricetin glycosides, and their aglycones, were detected at a wavelength of 360 nm, while anthocyanins were assayed at 520 nm. Standards of cyanidin-3-rutinoside, myricetin and kaempferol-3-glucoside were purchased from Extrasyntese Company (Genay, France); quercetin, kaempferol, quercetin-3 rutinoside and (-) epicatechin were purchased from Sigma-Aldrich (Poznań, Poland). To identify anthocyanins and the remaining flavonoids, standards were used, and UV-vis spectra was employed.

Animal study

The animal protocol used in this study was approved by the local Institutional Animal Care and Use Committee. The study was

	Blackcurrant pomace	
	Unprocessed	**Processed**
Chemical composition, %		
Dry matter	95.5 ± 0.0	90.0 ± 0.1
Crude protein	12.8 ± 0.3	12.5 ± 0.1
Crude fat	3.5 ± 0.2	3.3 ± 0.3
Crude ash	2.8 ± 0.0	2.1 ± 0.0
Total dietary fiber (TDF), including:	66.5 ± 0.8	67.6 ± 0.2
Soluble dietary fiber (SDF)	5.1 ± 0.2	3.4 ± 0.0
Nitrogen-free extract (NFE)	10.0 ± 1.2	4.4 ± 0.3
Polyphenolic compounds (HPLC-DAD), mg/100 g		
Total anthocyanins, including:	512.1 ± 18.9	119.7 ± 3.0
Delphinidin-3-rutinoside†	183.7 ± 6.6	55.3 ± 0.7
Delphinidin-3-glucoside†	156.6 ± 6.3	68.4 ± 1.0
Cyanidin-3-rutinoside†	110.1 ± 3.7	38.6 ± 0.8
Cyanidin-3-glucoside†	48.5 ± 2.0	20.1 ± 0.3
Other anhocyanins	13.3 ± 0.3	9.3 ± 0.1
Total flavonols aglycones, including:	65.5 ± 3.0	49.3 ± 0.2
Myricetin	42.0 ± 2.3	29.1 ± 0.1
Quercetin	17.6 ± 0.5	14.5 ± 0.1
Kaempferol	4.6 ± 0.0	4.3 ± 0.1
Isorhamnetin‡	1.3 ± 0.1	1.4 ± 0.0
Total flavonols glycosides, including:	20.4 ± 0.6	12.1 ± 0.0
Myricetin glycosides¶	10.3 ± 0.4	5.7 ± 0.0
Quercetin glycosides¶	5.3 ± 0.1	3.3 ± 0.0
Kaempferol glycosides¶	2.4 ± 0.2	1.6 ± 0.0
Isorhamnetin glycosides¶	2.3 ± 0.1	1.5 ± 0.0
Total polyphenols	598.0 ± 22.4	253.1 ± 3.2
HPLC-DAD, high-performance liquid chromatography with a diode array etector		
*Data are presented as means ± SD (n=3)		
†The content of the substance calculated on cyanidin-3-rutinoside		
‡The content of the substance calculated on quercetin		
¶The content of glycosides calculated on quercetin-3-rutinoside		

Table 1: Basic chemical (g/100 g) and polyphenolic (mg/100 g) composition of unprocessed and processed blackcurrant pomace*.

conducted on 20 male New Zealand white rabbits aged 34 d and weighing 630 ± 25 g, randomly divided into 4 groups of 5 animals each. They were kept individually in wire net flat deck cages and maintained under standard conditions: Temperature of 19-22°C, relative air humidity of 60-75%, intensive ventilation of rooms, and regulated photoperiod (16-h lighting and 8-h darkness). Feed and tap water were freely available. The detailed composition of the isonitrogenous diets is given in Table 2. For 4 weeks rabbits were subjected to following dietary treatments: groups SBR and SBL were fed standard chow with 15% of either unprocessed or processed blackcurrant pomace, respectively; groups HFBR and HFBL received lard-enriched diet with 15% of either unprocessed or processed pomace, respectively. In the standard diets the energy sources consisted 21% from protein, 7% from fat, and 72% from carbohydrates; in the fat-enriched diets these values were as follows: 17% from protein, 32% from fat, and 51% from carbohydrates.

Individual feed consumption and body weight (BW) gains of rabbits were determined. After the experiment, rabbits were weighed and anesthetized with sodium pentobarbitone according to recommendations for euthanasia of experimental animals. Blood samples were taken from jugular vein into test tubes, and then serum was prepared by solidification and low-speed centrifugation (350 × g, 10 min, 4°C). Animals were killed by cervical dislocation. After laparotomy, the selected tissues (liver, kidneys, and heart) were removed and weighed.

Lipid peroxidation products in the serum and tissue of the internal organs were assessed by reaction with tiobarbituric acid as TBARS, according to the method of Uchiyama and Michara [11]. The results are expressed as nanomoles of TBARS per gram of tissue or ml of serum. Serum samples were assessed for concentrations of glucose (GL) and lipids, including TC, HDL-cholesterol (HDL-C) and TAG, using direct-measurement assays (Alpha Diagnostic Ltd., Warsaw, Poland). The AI of a diet was calculated for each animal according to the formula AI=log (TAG/HDL-C). The amount of free fatty acids (FFA) present in serum of rabbits was assessed through a coupled reaction to measure non-esterified fatty acid with a commercially available detection kit (Serum/Plasma Non-Esterified Fatty Acids Detection Kit; Zen-Bio, Research Triangle Park, NC, USA). To measure insulin concentration, a validated rabbit insulin ELISA kit was used (Dog, Human and Rabbit Insulin Elisa Kit; Kamiya Biomedical Company, Seattle, USA). Homeostasis model assessments for insulin resistance (HOMA-IR) and pancreatic insulin secretion (HOMA-β) were calculated according to the following formulas: HOMA-IR=[fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5], while HOMA-β=[fasting insulin (mU/l) × 20/ fasting glucose (mmol/l) – 3.5]. TAS of serum was measured using two-reagent assay (TAS Kit; Randox Laboratories Ltd., Crumlin, United Kingdom). The serum ACW and ACL were determined by a photochemiluminescence detection method, using a Photochem analyzer (ACW-Kit and ACL-Kit; Analytik Jena AG, Jena, Germany). Ascorbate and Trolox calibration curves were used in order to evaluate ACW and ACL, respectively, and the results were expressed as mmol ascorbate or Trolox equivalent/ml serum.

Statistical analysis

Values are expressed as means ± SD. The STATISTICA software, version 8.0 (StatSoft Corp., Krakow, Poland), was used to determine whether variables differed among treatment groups. Two-way ANOVA was applied to assess the effects of blackcurrant product processing (unprocessed and processed pomaces differed substantially in polyphenols level), type of diet (standard chow and chow with additional dietary lard) and the interaction between investigated factors (type of supplement × type of diet). When the ANOVA indicated significant treatment effects, means were evaluated using Duncan's multiple range test. Data were checked for normality before statistical analysis was performed. Differences with $P \leq 0.05$ were considered to be significant.

Results

The initial BW of rabbits did not differ between groups (data not shown). During 4 week of feeding, BW gain and daily diet intake

	Group			
	SBR	**SBL**	**HFBR**	**HFBL**
Component, g/100 g of a diet				
Oat	14	14	14	14
Wheat bran	10	10	10	10
Sunflower meal	16.8	16.8	16.8	16.8
Dried sugar beet pulp	5	5	5	5
Grass meal	16	16	1	1
Barley	16.1	16.1	18.1	18.1
Soybean meal	4.1	4.1	7.1	7.1
Unprocessed blackcurrant pomace	15	-	15	-
Processed blackcurrant pomace	-	15	-	15
Lard	-	-	10	10
Additives*	3	3	3	3
Calculated chemical composition, %				
Crude protein	17.5	17.5	17.4	17.4
Crude firber	14.3	14.3	12.6	12.6
Crude fat	2.4	2.4	13.6	13.6
Polyphenols†	0.09	0.04	0.09	0.04

*Per kg of feed: DL-methionine-2 g; L-lysine-2 g; Monocalcium phosphate 22.6%-11 g; Salt-3 g; Vitamin-mineral premix (5 g/kg) providing the following nutrients per kg feed: Vitamin A-10000 IU; Vitamin D-1800 IU, Vitamin E-15 mg; Vitamin K-4.5 mg; Vitamin B1-0.5 mg; Vitamin B2-4 mg; Vitamin B12-0.01 mg; Folic acid-0.1 mg; Pantothenic acid-7 mg; Nicotinic acid-20 mg; I-1 mg; Mn-60 mg; Cu-5.5 mg; Zn-75 mg; Fe-40 mg; Co-0.3 mg; Se-0.08 mg
†Polyphenols originated from blackcurrant pomaces

Table 2: Ingredients (g/100 g) and composition (%) of experimental diets.

increased significantly (P<0.005) in both groups fed diets with 10% of additional fat (Table 3). Final BW was also elevated in HFD-fed animals. The type of blackcurrant pomace had influence neither on weight gains nor on dietary intakes; however, rabbits receiving BL showed tendency to have decreased BW at the end of the experiment (P=0.088).

Irrespective of the polyphenol level in blackcurrant preparations, feeding with HFD caused a significant increase in the relative liver mass ($P \leq 0.001$), and at the same time decreased the relative heart mass (P<0.05), as compared to rabbits receiving standard chow (Table 3). The liver mass was significantly lower in animals given diets with BR pomace than in those fed the BL diets (P<0.05). TBARS concentration in all analyzed tissues (liver, kidney, heart, serum) was higher (P<0.05) in groups on fat-diets in comparison to animals on diets without additional fat. The level of polyphenolic compounds in blackcurrant pomace significantly affected the liver TBARS concentration (BL>BR, P<0.05). Similar effect was observed in the kidney's tissue (P<0.05). For serum concentration of TBARS, the two-way ANOVA showed a significant interaction B × D (P<0.05); in rabbits fed HFD, the dietary addition of BL significantly increased serum TBARS level in HFBL group, compared to HFBR group. No such effect was found for both groups fed standard diet. Regardless of the pomace type, additional fat in the diet caused a significant decrease in the level of serum TAS ($P \leq 0.001$), as well as ACW (P<0.005). Higher values for TAS and ACW were found when rabbits were fed the diet supplemented with polyphenol-rich pomace (P<0.05 for TAS, and $P \leq 0.001$ for ACW). The highest level of ACL was measured in the serum of rabbits fed SBR diet (P<0.05 vs. other treatments), while the lowest in both groups of rabbits fed diets supplemented with BL (see significant interaction B × D).

Serum levels of TAG, TC, insulin and both calculated HOMA indexes were significantly higher in the HFBL rabbits, as compared with other dietary treatments (Table 4). The addition of BR pomace to the HFD significantly reduced the concentrations of TAG, TC, insulin, HOMA-IR and HOMA-β (HFBL>HFBR, P<0.05), but the lowest values for these parameters were observed in both groups fed standard diet (see significant interaction B × D). In comparison to rabbits fed standard diets, feeding with HFD led to a significant increase in the AI value (P<0.05) and serum concentration of circulating FFA (P<0.001), as well as to a significant decrease in the HDL-C/TC profile ($P \leq 0.001$) in rabbits. The presence of BL in both types of diet led to a significant increase in the value of serum AI (P<0.05), as well as a tendency towards lower serum HDL-C/TC profile (P=0.076) in rabbits, in comparison to animals fed diets supplemented with unprocessed pomace, abundant in polyphenols.

Discussion

Hepatic steatosis associated with excessive fat accumulation in hepatocytes, is a condition often present in pathological states related to altered metabolism of the whole organism, which itself may contribute to other illnesses. In the current experiment, dietary administration of blackcurrant polyphenols was accompanied with reduced liver weight in HFD rabbits. As phytochemicals are metabolized by the liver, the organ is directly exposed to these compounds or their metabolic derivatives. Liver weight loss observed in this study may be associated with decreased hepatic fat accumulation and/or storage. In a 12-week experiment on C57BL/6J mice, supplementation of HFD (58% kcal from fat) with rich in cyanidin-3-glucoside extract of purple corn (0.02% of the diet), significantly decreased BW, concentration of TAG, as well as mRNA expression level of genes involved in fatty acid synthesis in the liver [12]. Similar results were obtained in the experiment of Cefalu et al. [13], in which 6 weeks of feeding with HFD supplemented with grape extract containing anthocyanin glycosides, prevented hypertrophy and accumulation of TAG in the liver of mice with genetically induced predisposition to obesity. In the present experiment, cyanidin-3-glucoside present in the diets containing unprocessed pomace constituted 0.007% of the diet, while all the anthocyanins -0.08%. It can therefore be assumed that a significant decrease in the liver weight (g/kg) of rabbits fed diets with unprocessed blackcurrant preparation, as compared to the groups fed diets with processed one, was due to the limited synthesis and accumulation of lipids in hepatocytes resulting from anthocyanin bioactivity.

	Dietary treatments*, n=5				*P* value		
	SBR	SBL	HFBR	HFBL	Pomace (B), n=10	Diet (D), n=10	B × D
Final BW, kg	1.98 ± 0.27	1.78 ± 0.13	2.24 ± 0.13	2.14 ± 0.15	NS	<0.005	NS
BW gain, kg/4 weeks	1.36 ± 0.28	1.16 ± 0.14	1.61 ± 0.13	1.52 ± 0.14	NS	<0.005	NS
Diet intake, g/day	102 ± 14	94 ± 10	114 ± 4	114 ± 8	NS	<0.005	NS
Internal tissues							
Liver mass, g/kg of BW	24.6 ± 3.2	26.7 ± 3.3	28.9 ± 2.1	34.8 ± 4.3	<0.05	≤0.001	NS
TBARS, nmol/g of tissue	73.8 ± 4.0	75.9 ± 3.6	85.7 ± 6.1	91.8 ± 2.3	<0.05	<0.001	NS
Kidney mass, g/kg of BW	6.18 ± 0.22	6.32 ± 0.71	6.36 ± 1.90	6.14 ± 0.47	NS	NS	NS
TBARS, nmol/g of tissue	112 ± 7	117 ± 11	129 ± 8	146 ± 14	<0.05	<0.001	NS
Heart, g/kg of BW	2.53 ± 0.21	2.69 ± 0.43	2.28 ± 0.13	2.39 ± 0.21	NS	<0.05	NS
TBARS, nmol/g of tissue	64.0 ± 3.1	63.7 ± 3.3	66.5 ± 3.7	69.1 ± 2.4	NS	<0.05	NS
Serum							
TBARS, nmol/ml	45.4 ± 3.4^{c}	50.4 ± 3.2^{c}	107 ± 14^{b}	128 ± 13^{a}	≤0.01	<0.001	<0.05
TAS, mmol/l	1.26 ± 0.12	1.17 ± 0.06	1.10 ± 0.08	1.03 ± 0.51	<0.05	≤0.001	NS
ACW, mmol AA/ml	0.081 ± 0.006	0.071 ± 0.006	0.072 ± 0.005	0.061 ± 0.005	≤0.001	<0.005	NS
ACL, mmol Trolox/ml	0.099 ± 0.002^{a}	0.092 ± 0.004^{c}	0.094 ± 0.004^{b}	0.092 ± 0.007^{c}	<0.05	NS	<0.05

ACW: Antioxidant capacity of water-soluble substances in the serum; ACL: Antioxidant capacity of lipid-soluble substances in the serum; BW: Body weight; NS: Not statistically significant; TAS: Total antioxidant capacity; TBARS: Thiobarbituric acid-reactive substances

*Groups SBR and SBL fed standard chow with 15% unprocessed and processed blackcurrant pomace, respectively; groups HFBR and HFBL fed lard-enriched diet with 15% unprocessed and processed pomace, respectively (means ± SD, n=5)

^{a,b,c}Mean values within a row with unlike superscript letters were shown to be significantly different ($P \leq 0.05$) in the case of a statistically significant interaction B × D ($P \leq 0.05$)

Table 3: Body weight and parameters of antioxidant status in rabbits fed experimental diets.

	Dietary treatments†, n=5				*P* value		
	SBR	SBL	HFBR	HFBL	Pomace (B), n=10	Diet (D), n=10	B × D
GL, mmol/l	6.03 ± 0.76	6.11 ± 0.91	5.96 ± 0.32	5.88 ± 0.60	NS	NS	NS
TAG, mmol/l	1.10 ± 0.20^{c}	1.02 ± 0.23^{c}	1.26 ± 0.13^{b}	1.44 ± 0.25^{a}	NS	<0.01	<0.05
TC, mmol/l	2.02 ± 0.26^{c}	1.92 ± 0.23^{c}	2.28 ± 0.32^{b}	2.55 ± 0.28^{a}	NS	<0.005	<0.05
HDL-C, mmol/l	1.08 ± 0.14	0.98 ± 0.17	1.06 ± 0.15	1.04 ± 0.11	NS	NS	NS
HDL-C/TC, %	53.8 ± 4.2	51.0 ± 3.7	46.5 ± 5.2	41.2 ± 5.3	0.076	≤0.001	NS
AI‡	0.004 ± 0.111	0.013 ± 0.131	0.077 ± 0.081	0.136 ± 0.039	<0.05	<0.05	NS
FFA, mmol/l	425 ± 127	402 ± 111	1002 ± 119	1110 ± 247	NS	<0.001	NS
Insulin, pmol/l	54.0 ± 2.5^{c}	55.6 ± 2.3^{c}	67.1 ± 1.6^{b}	77.9 ± 1.6^{a}	0.088	<0.001	<0.05
HOMA-IR¶	2.08 ± 0.5^{c}	2.17 ± 0.3^{c}	2.56 ± 0.6^{b}	2.93 ± 0.6^{a}	0.062	<0.05	≤0.05
HOMA-β§	61.5 ± 3.5^{c}	61.3 ± 4.4^{c}	78.5 ± 6.9^{b}	94.3 ± 3.6^{a}	0.072	≤0.05	<0.05

AI: Atherogenic index; FFA: Free fatty acids; GL: Glucose; HDL-C: HDL cholesterol; HOMA-IR: Homeostasis model assessment for insulin resistance; HOMA-β: Homeostasis model assessment for pancreatic insulin secretion; NS: Not statistically significant; TAG: Triacylglyceride; TBARS: Thiobarbituric acid-reactive substances; TC: Total cholesterol

*Blood samples were taken in overnight food-deprived animals

†Groups SBR and SBL fed standard chow with 15% unprocessed and processed blackcurrant pomace, respectively; groups HFBR and HFBL fed lard-enriched diet with 15% unprocessed and processed pomace, respectively (means ± SD, n=5).

‡AI=log(TAG/HDL-C)

¶HOMA-IR=[fasting insulin (mU/l) × fasting glucose (mmol/l)/22.5]

§HOMA-β=[fasting insulin (mU/l) × 20/fasting glucose (mmol/l)-3.5]

^{a,b,c} Mean values within a row with unlike superscript letters were shown to be significantly different ($P \leq 0.05$) in the case of a statistically significant interaction B × D ($P \leq 0.05$)

Table 4: Biochemical indices of the serum in experimental rabbits*.

It is well established that elevated levels of TAG and LDL-cholesterol (LDL-C) are risk factors for cardiovascular diseases and serum concentration of HDL-C is inversely related to that risk. In dyslipidemic patients, pure anthocyanins derived from bilberry and blackcurrant produced a dual beneficial effect on lipoprotein profile, which included a decrease in LDL-C and an increase in HDL-C serum levels [14]. In the present experiment different dietary treatments did not affect HDL-C concentration in rabbits' serum; however, among HFD-fed animals, supplementation of the diet with BR decreased elevated HDL-C/TC profile. In these animals, beneficial changes were also observed for the serum level of TAG, TC and calculated value of AI index. In numerous studies on animal models, as well as those using in vitro systems, attempts have been made to clarify the possible mechanisms involved in the regulatory effect exerted by polyphenols from berries on the absorption and metabolism of lipids. It is suggested that the hypolipidemic effect of dietary polyphenols may be associated with the inhibition of lipid absorption from the gastrointestinal tract. It was shown that polyphenols from berries have the ability to inhibit the activity of lipolitic enzymes (including pancreatic lipase), which greatly handicap the uptake of fat from the intestinal lumen [12].

Results of the present study demonstrate that polyphenols from blackcurrant pomace exert advantageous influence on insulin secretion and action, reducing dietary-induced hyperinsulinemia as well as pancreatic insulin secretion and systemic insulin resistance (manifested by lower HOMA-β and HOMA-IR values, respectively). Within the animals fed a diet with 10% fat content, rabbits, whose diet was enriched with unprocessed blackcurrant preparation, compared to HFBL group, had lower values of all aforementioned biomarkers in the serum at the end of the experiment. This indicates the potential use of blackcurrant polyphenols in the control of insulinemia. Insulin resistance is often proposed to be a major causative factor in fatty liver development. Due to the fact that, in comparison to the animals fed processed pomace, rabbits fed diets supplemented with BR had most likely lower fat accumulation in their livers (which was indirectly demonstrated by the lower weight of this organ in SBR and HFBR groups), as well as preferable characteristic of serum lipids, possible regulatory effect of blackcurrant polyphenols on lipid absorption and/or metabolism may account for an improved insulinemia observed in these animals. Polyphenolic compounds, especially anthocyanins, seem to influence the cascade of reactions associated with insulin secretion and signaling [12,13,15].

Free radicals and products of their reaction with biomolecules are the leading risk factors for developing atherosclerosis, some cancers, as well as accelerated aging processes [16,17]. The severity of their appearance leads to a condition called oxidative stress and it has been postulated for a long time that the health-promoting activity of phenolic compounds mainly relays on their ability to reduce oxidative stress. Polyphenols are potent scavengers of reactive forms of oxygen and nitrogen, able to inhibit the activity of enzymes and chelate metal ions involved in catalyzation of oxidation reactions [18]. Although ORAC analysis showed that the antioxidant capacity of lipophilic antioxidants derived from berries is rather small [19], numerous studies have demonstrated beneficial effects of whole berries or polyphenolic fraction extracted from these fruits on oxidation of blood or internal organ lipids [9,10,20,21]. In the current study, blackcurrant polyphenols were shown to inhibit lipid peroxidation induced by dietary fat (lower level of TBARS in the liver, kidneys and serum as well as serum ACL in rabbits from HFBR, compared to HFBL group). The suppression of lipid peroxidation observed in the liver and kidneys of rabbits fed diet enriched with unprocessed pomace, is probably the effect of polyphenolic compounds that may increase filtration in these organs and inactivate free radicals. The significantly higher overall activity of endogenous antioxidant capacity observed in serum of rabbits fed diet with natural pomace (demonstrated by higher TAS, ACW and ACL values), may suggest that large amount of dietary polyphenols exerted stimulating and/or protective effect towards the activity of endogenic antioxidant compounds found in serum (i.e., glutathione, vitamin E). It was shown that polyphenols may interact synergistically with other antioxidants (both endogenous and delivered with a diet), thereby affecting the final antioxidant effect [22,23]. It should be noted that dietary fiber may also influence the antioxidant properties of phenolic compounds, i.e., by providing protection for the bioactive substances against the acidic environment of the stomach and/or digestive enzymes [4].

Our study showed that the higher content of polyphenolic compounds in the diet preferably affected the antioxidant status and blood lipid profile of rabbits. In order to affect the function of cells and tissues beyond the gastrointestinal tract, bioactive components of the diet need to be absorbed and reach the peripheral circulation. It has been postulated that delivering polyphenolics together as a complex with dietary fiber in natural products is a good approach enabling utilization of antioxidant properties of phenolic compounds by the intestinal flora. Colonic fermentation of these both constituents yields physiologically active metabolites with potential systemic effects. Apart from polyphenols, both preparations tested in the present study contained reasonable amounts of dietary fiber. An additive or even synergistic effect of dietary fiber and polyphenols in counteracting poor metabolic outcomes caused by the HFD is highly possible, and cannot be excluded when interpreting the results of the study.

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Psychological Bias Caused by Notification of Brand Name in Sensory Evaluation of Mango Fruit Drink

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Abstract

Consumer buying habit of food product is driven by brand name. The objective of this study is to examine whether brand name have an effect on the evaluation of sensory-based attributes in the process of purchasing a product. Brand name plays an important role even it causes psychological bias in sensory evaluation of mango fruit drink. In this study we conducted five different sensory test-blind test, open test, interchange sample test, duplicate test and brand recognition test to check the bias caused by notification of brand name. Same respondents were evaluated for all the tests. Sensory evaluation was done on attributes like color, smell, taste, sweetness, mouth feel, viscosity and overall acceptance using the 1-9 hedonic scale. Tests results proved that brand name does cause bias during sensory evaluation of mango fruit drink. People knew mango drinks by their brand name and not by their taste or product quality. More successful the brand more rating it got during open and interchange sample test. After conducting the test it is observed that different product may taste more or less the same but brand name is unique which guides the sensory evaluation. People were not able to identify the brand by tasting it in brand recognition test. On an average only 20% of the consumers successfully identified the brand by taste.

Keywords: Sensory test; Brand name; Hedonic scale; Psychological bias

Introduction

Today's consumers are discerning, demanding and becoming more knowledgeable about food and beverages. They accept only products which are safe, value added and of high sensory quality [1]. Therefore, knowing consumers preferences and perceptions about the sensory characteristics of food products is very important to food and beverage manufacturers and retailers alike. Now-a-days customers have a good knowledge about the branded products and they trust more the well-known brands assuming they offer them good quality that they expect [2,3]. Brand name greatly affects the sensory result of the products [4-6]. A brand value depends on the quality of its products in the market and the need that a customer actually got satisfied with by using its products and services [7]. This builds the trust of the customers on that brand [8]. Customer loyalty is the result of consistently positive emotional experience, physical attribute-based satisfaction and perceived value of an experience, which includes the product or services provided by a company [9,10]. Customers trust is valuable asset for a company because it reduces vulnerability from competitors [7]. Customer decision making process is highly influenced by brand name in most of the cases.

Sensory evaluation of product is carried out to determine consumer acceptability [11]. Sensory Evaluation is defined as "A scientific discipline used to evoke, measure, analyze, and interpret those responses to products that are perceived by the senses of sight, smell, touch, taste, and hearing". The most widely used scale for measuring food acceptability is the 9-point hedonic scale (Table 1) [12]. David Peryam and colleagues developed the scale at the Quartermaster Food and Container Institute of the U.S. Armed Forces, for the purpose of measuring the food preferences of soldiers [13]. The sensory attributes for a fruit drink on which rating is given are Color, smell, taste, mouth-feel, sweetness, viscosity and overall acceptance [13]. Psychological factors affecting sensory test are Expectation error which occurs when an individual is given too much information about the samples, Stimulus error which occurs when an individual is influenced by some characteristics of the sample, Halo effect which occurs when an individual rate more than one quality characteristics at a time, Suggestion error which occurs when an individual is aware of the reactions of other during sensory evaluation, Central tendency error which occurs when the individual may choose the mid-range to avoid extremes and Order effect error which occurs when samples are placed in a defined order. Knowing the brand name would also cause psychology bias during sensory evaluation of a product which comes under expectation error [14-16]. A study showed how brand name influence consumer decision of buying cars [17]. Best method to avoid expectation error is to do blind sensory test. In this paper we have tried to find out and prove that consumers are biased towards well-known brands.

For this study we have chosen six locally available mango fruit

Score/rating	std. hedonic scale
9	I like extremely
8	I like very much
7	I like moderately
6	I like sightly
5	I neither like or dislike
4	I dislike slightly
3	I dislike moderately
2	I dislike very much
1	I dislike extremely

Table 1: 9 Point hedonic scale.

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drink. Fruit drink is the largest market with an estimated market share of close 50% -60%, of which mango is the most popular flavor and accounts for 90% of volume across all the variants [18].

Methodology Used

Survey Method has been chosen to carry out the study. Similar study has been done on wine [19].

Respondents

A total of 80 respondents were taken as sample. These respondents are consumers of mango fruit drink. All the respondents were taken from organization-Flourish Pure foods Pvt. Ltd., Ahmedabad. Age group was from 20-50. Sensory sampling was done on the same respondents for all the tests. Each day all the respondents were taken for single test. Each time sealed pack of mango beverage was used. No restrictions were there on age, gender and location. All the respondents were physically and mentally fit. Neither of them had any undesired medical history which can influence the test result. All respondents before this study started had tried all the six mango beverages.

Mango beverage

We have used six different brands which were locally available in the market.

Sensory analysis

Sensory evaluation of the fruit drink was judged for color, taste, flavor, smell, viscosity and general acceptability on a nine point hedonic scale, varying from "dislike extremely" (Score 1) to "like extremely" (Score 9) was used according to [14]. The whole survey was divided into five tests: Blind sensory test, Open sensory test, Interchange sample test, Duplicate sensory Test and Brand recognition test [20-22].

We conducted five different tests to know the psychological bias that an individual does during sensory evaluation [23]:

- Blind sensory test of mango fruit drink of 6 different brands: In this test respondents were given 50ml of unknown mango fruit drink. The mango fruit drink were named as Brand 1, Brand 2, Brand 3, Brand 4, Brand 5 and Brand 6. They were asked to drink and fill the sensory evaluation form on different parameters like color, odor, taste, mouth-feel, sweetness, viscosity and overall acceptability using hedonic scale.

- Open sensory test of mango drink of 6 different brands: In this test the respondents knew which brand mango fruit drink they are going to evaluate. In this test the same respondents were given the same quantity of all the 6 brands of mango fruit drink. They were asked to evaluate on the same parameter as in blind taste.

- Interchanging pack test: In this test packs of different mango drink brands were interchanged. In brand 1 pack brand 3 mango fruit drink was filled and vice versus. In brand 2 pack brand 4 mango fruit drink was filled and vice versus. Brand 5 and Brand 6 were left unchanged. The same respondents were asked to fill the sensory evaluation form on same parameters.

- Duplicate Sensory Test of mango fruit drink: In this test, two brand samples were kept in duplicate. The respondents were given 8 samples to taste one after one and rate and asked them to rate using hedonic scale. Brand 1 and Brand 2 were kept in duplicate.

- Brand Recognition test: In this test respondents were given mango fruit drink and asked to identify the brand according to taste.

Limitation of the study

- Only limited numbers of respondents were tested.
- The test had no restriction of age.

Results and Discussion

Blind sensory test result

Blind sensory test result is most accurate and unbiased. For our study it will act as control or reference. This test result tells about consumer acceptability of particular food product. Respondents were asked to rate the samples using 1-9 hedonic scale on different attributes like color, odor, taste, mouth-feel, sweetness, viscosity and overall acceptance (Tables 2 and 3).

The blind sensory test result states that: Brand 1 mango fruit drink ranked 1st in all the parameters.

Brand 2 ranked 2nd in taste, mouth-feel and overall, ranked 3rd in color, smell, sweetness and viscosity. As this test is unbiased this test ranking is most accurate and would be treated as reference.

Open sensory test of mango fruit drink

This test is for analysis of biasing that respondents will do on knowing the information the pack provides. The respondents filled the same sensory evaluation table but this time they knew the brand which they were consuming (Tables 4 and 5).

Open sensory result shows that: Brand 1 and Brand 2 is still at 1st and 2nd position respectively But Brand 5 which ranked 5th in blind test ranked 3rd in open test which was surprising to note. The brands whose ranking changed, signifies that respondents had judged the mango fruit drink of less quality when they saw its label. The only difference between open and blind test is of the additional information the brand provides to respondents (Tables 6 and 7).

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Overall	1	2	3	4	5	6
Color	1	3	4	2	6	5
Smell	1	3	2	5	4	6
Taste	1	2	3	4	5	6
Mouth-feel	1	2	4	3	6	5
Sweetness	1	3	2	5	6	4
Viscosity	1	3	2	5	4	6

Table 2: Ranking list of blind test.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Total avg. points	7.21 ± 1.0997	6.27 ± 0.998	6.22 ± 0.389	6.15 ± 0.838	5.96 ± 0.551	4.96 ± 0.947
Ranking	1	2	3	4	5	6

Table 3: Average points of all the attributes for blind test.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	BRAND 6
Overall	1	2	5	4	3	6
Color	1	2	3	6	5	4
Smell	1	2	4	5	3	6
Taste	1	2	5	3	4	6
Mouth-feel	1	2	4	3	3	5
Sweetness	1	2	4	4	3	5
Viscosity	1	2	5	3	4	6

Table 4: Ranking list of open test.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Total avg. points	7.12 ± 1.002	6.73 ± 0.881	6.28 ± 0.403	6.09 ± 0.930	6.38 ± 1.11	5.73 ± 0.362
Ranking	1	2	4	5	3	6

Table 5: Average points of all the attributes for open test.

Pack	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Fruit Drink	Brand 3	Brand 4	Brand 1	Brand 2	Brand 5	Brand 6
Overall	2	3	1	6	5	4
Color	3	2	1	6	5	4
Smell	1	3	1	4	2	5
Taste	2	1	3	5	1	4
Mouth-feel	3	1	2	4	3	4
Sweetness	1	2	1	4	3	3
Viscosity	2	4	1	6	5	3

Table 6: Ranking list of interchange pack test.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Total avg. points	6.75 ± 0.248	6.29 ± 0.999	6.6 ± 0.916	6.57 ± 0.535	6.4 ± 0.721	6.3 ± 0.458
Ranking	1	6	2	3	4	5

Table 7: Average points of all the attributes for interchange test.

Interchanging pack test

Packs were interchanged and respondents were asked to fill the form. Ranking: Brand 1 ranked 1st, Brand 2 ranked 6th (Table 8).

Comparison of open test, blind test and interchange pack test

Brand 1 fruit drink ranked 1st in all the three tests', Brand 1 has worked effectively on the quality of fruit drink and has done right branding of the mango fruit drink. This is ideal behavior which ever other brand should do. Respondents were not biased when judging Brand 1.

Brand 2 fruit drink ranked 2nd in blind test(Reference test) and Open test(Analysis Test) because brand 2 was a well-known brand but brand 2 fruit drink ranked 3rd when its pack was interchanged highlighting the biasing which respondents did when the pack was changed with less famous brand.

Brand 3 fruit drink ranked 3rd in Blind test but when respondents were shown the pack it ranked 5th it this clearly signifies the biasing which respondents did on seeing the pack. Similarly, for Brand 5 and Brand 6 the result was same. Biasing of respondents was there.

Brand 4 fruit drink ranked 4th in blind test which is control but on seeing the pack of the brand it ranked 3rd and in third test when the pack was interchanged with a not so famous brand it ranked 5th. These results clearly highlights the biasing which respondents did during the tests.

Brand 6 fruit drink ranked 6th in blind and open test but randomly ranked 2nd in interchange sample test even when its pack was not change. This Random result drives us to conduct duplicate test to examine the variation respondents will do when the samples are kept in duplicate. All these results clearly show that respondent's sensory preference is driven by the brand name of the mango fruit drink (Table 9).

Duplicate test

In this test Brand 1 and Brand 2 was kept in duplicate. All the 8 fruit drink were rated on same different attributes using hedonic scale.

Respondents rated Brand 1 and Brand 2 differently both the times which shows that they were not able to identify that the fruit drink were same. The respondents use their brain not their senses. The two fruit drink got different rates despite being the same. They considered all the fruit drink to be different. The average rate which Brand 1 got in first turn is 6.95 and in second turn it got 6.50 showing a variation of 0.45. Similarly, for Brand 2 in first it got 6.45 average rates while its duplicate fruit drink got 5.95 showing a variation of 0.50 (Figures 1 and 2) (Table 10).

Brand recognition test

This test is also perceptual discrimination tests**.** In this test we wanted to find out whether the respondents are able to identify the taste, odor, mouth feel of the brands which they were consuming. In other words, the goal of the test is to determine whether the respondents can sense the dissimilarities between different brands. The results were surprising, for every brand on an average only 20% of the consumers identified the brand by tasting it (Figure 3).

- Brand 1: Only 30% of respondents identified the brand correctly. This percentage is very low, it is expected that people should have identified it correctly, because Brand 1 ranked 1st in the entire above test.
- Brand 2: Only 20% identified the brand correctly which shocking as Brand 2 is a well-known brand and is enjoying a huge market share.
- Brand 3: Only 10% identified it correctly.
- Brand 4: Only 20% identified it correctly.
- Brand 5:0% of the respondents identified the brand. Brand 5 was a brand known for its taste.
- Brand 6: Only 20% of the respondents identified it correctly.

Ranking	1	2	3	4	5	6
Blind	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Open	Brand 1	Brand 2	Brand 4	Brand 5	Brand 3	Brand 6
Interchange	Brand 1	Brand 6	Brand 2	Brand 3	Brand 4	Brand 5

Table 8: Comparison of all the tests.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 4	Brand 5	Brand 6
Blind Test Avg	7.21 ± 1.09	6.27 ± 0.99	6.22 ± 0.38	6.15 ± 0.83	5.96 ± 0.55	4.96 ± 0.94
Open Test Avg	7.12 ± 1.00	6.73 ± 0.88	6.28 ± 0.40	6.09 ± 0.93	6.38 ± 1.11	5.73 ± 0.36
Interchange Test Avg	6.75 ± 0.24	6.29 ± 0.99	6.6 ± 0.91	6.57 ± 0.53	6.4 ± 0.72	6.3 ± 0.45

Table 9: Comparison of all the tests.

Fruit drink	Brand 1	Brand 2	Brand 3	Brand 1	Brand 4	Brand 5	Brand 6	Brand 2
Average	6.95 ± 1.27	6.45 ± 0.47	6.5 ± 0.80	6.5 ± 0.50	6.3 ± 0.78	6.6 ± 0.50	6 ± 1.34	5.95 ± 0.79

Table 10: Average points of each brand in duplicate test.

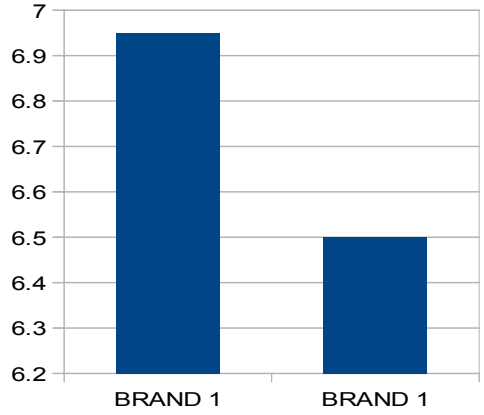

Figure 1: Variation is of 0.45.

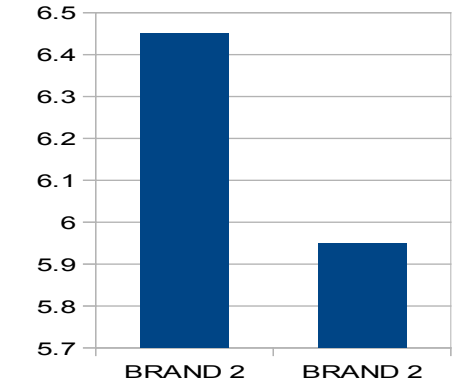

Figure 2: Variation is of 0.50

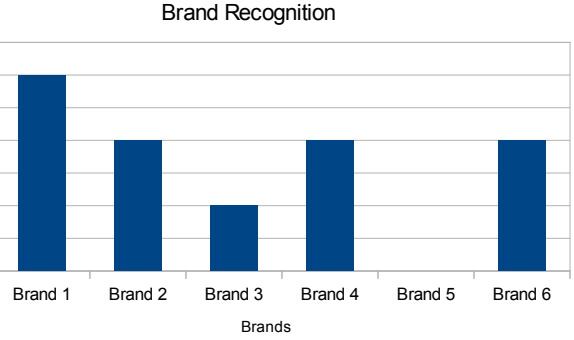

Figure 3: % Brand recognition chart.

All these results proof that respondents actually do not know the mango drink by taste they know the drink only by the brand name. Only few respondents were able identify the mango fruit drink brand correctly. So only brand name plays an important role in the market and among the respondents because respondents get biased towards a well-known brand. If a new player wants to enter the market it has to pay special attention towards branding if it wants to enjoy a major position in the market.

Conclusion

The result of every test proves that brand name plays an important role in sensory evaluation because consumers tend to get biased towards a well-known brand as they trust the brand. There is tendency to rate higher a famous brand in all the attributes. If a food and beverage manufacturer wants to get unbiased result it should conduct blind test of the samples at least 3 times (to remove duplicity deviation) and use the average as the final result. This result will true and unbiased. It is also being concluded that to be successful in market product quality as well as brand image plays an important role.

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Quantitative Analysis of Shilajit using Laser-Induced Breakdown Spectroscopy and Inductively Coupled Plasma/Optical Emission Spectroscopy

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Abstract

Laser-induced Breakdown Spectroscopy (LIBS) was used for the quantitative study of Shilajit samples using first harmonic (1064 nm) of a Q-Switched Nd:YAG laser by an appropriate detection system. The samples were chosen from the northern Himalayan mountainous range at locations namely Skardu, Gilgit, Chilas, Chitral, and Muzaffarabad. The relative concentrations of detected species were measured via integrated intensity of the strongest line of an element and via Calibration-Free CF-LIBS approach. Our findings recommend the altitudinal variations in Physico-chemical properties of Shilajit. Different functional groups were identified using Fourier Transform infrared (FTIR)-Spectroscopy. The results of LIBS were compared to results of Inductively Coupled Plasma/Optical Emission Spectroscopy (ICP/OES) with fine harmony.

Keywords: Laser-induced Breakdown Spectroscopy (LIBS); Shilajit plasma; Edible analysis; Quantitative study

Introduction

Laser-induced Breakdown Spectroscopy (LIBS) is used extensively for the quantitative as well as qualitative study of different materials [1-4]. Whenever laser pulses are focused on a solid target surface, breakdown of the laser irradiated part of target occurs. Based on the properties of laser pulse, for example, energy (E), wavelength (λ), pulse duration (tp) etc., and on the physical and chemical properties of the specimen, the laser removal removes the amount of test sample fluctuating from several Nano-grams to micro-grams. At the point when nanosecond laser pulses are utilized, electrons gain a considerable segment of the pulse thereby increasing the kinetic energy. Under these circumstances hot and dense expanding plasma is generated. The radiation emitted from such plasma provides a distinctive spectral signature that facilitates the detection of elemental composition of the target. The intensities of spectral emission lines observed in the spectrum can be used to measure the relative abundance of species in the sample [5]. Literature survey report the significant work using LIBS on the elemental analysis of materials including marbles [6,7], metals and ceramics [8], vegetables [9], crystals [10], pigments [11], and plant materials [12,13]. This method has been used for the elemental analysis fertilizers [14]. Laser-induced breakdown spectroscopy has applied successfully for quantitative analysis of calcified tissue samples [15]. The detection of chromium has also been performed using LIBS [16]. This technique is found capable for the detection of Cu, Pb, V and Zn in polluted soil [17]. LIBS in conjunction with Inductively Coupled Plasma-Optical Emission Spectroscopy (ICP-OES) have been applied to determine lead in paints [18].

The present work was performed to utilize LIBS to analyze the elemental composition and relative concentration of the detected species in Shilajit occurring in Pakistan, which is used in as medicine to increase endurance, strength, and immunity. The Shilajit samples were collected from the northern Himalayan mountainous range at different locations namely Skardu, Gilgit, Chilas, Chitral, and Muzaffarabad, were converted in pellets that were exposed to laser light to record the emission spectra. The relative concentrations of the detected species in the samples were determined by integrated intensity of strongest line of an element and by calibration-free LIBS method and propose the altitudinal variation in Physico-chemical properties of Shilajit, which will be useful for its fruitful treatment in medicines for diverse maladies. Spectral analysis technique of FTIR was used to study different functional groups. The results of LIBS were compared to the outcomes of ICP/OES and were found in sound conformity. The results by means of ICP/OES were more perfect; but this methodology needed more time and sample preparation, while LIBS is easy and the results can be obtained by minimum sample preparation.

Experimental Setup

The experimental setup used is exposed in Figure 1. The laser pulse was produced using a custom made at 1064 nm of Q-switched Nd:YAG laser that was optimized to deliver 100 mJ/ pulse with a time delay of 0.4 μs. The plasmas were generated by focusing a pulsed laser onto the surface of the target placed atmospheric pressure. The laser beam was focused on the target using a Plano-convex lens having a focal length of 20 cm and diameter 30 mm. The samples were pasted on a rotating holder that was moving uniformly at the rate 6.1 mm/s using a step motor to in order to provide a fresh target surface for every laser shot and to avoid the non-uniform pitting of the target surfaces. In order to avoid ambient gas breakdown, we have gone through many pre- tests to find out the optimum point for placing the focusing lens. The distance between focusing lens and target was optimized by investigating the effect of its separation on the quality of spectra. In our case it was ~18 cm.

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The emitted radiations from the plasma were collected by a fiber optics (high-OH, core diameter: 600 μm) having a collimating lens (0°–45° field of view) placed perpendicular to the direction of laser beam. From the spot diameter of ~102 μm, laser fluence was approximated to be 40 J/cm^2. The pulse energy was measured with a calibrated Joule meter (Quantel NOVA-QTL, USA). The light radiated from plasma flash was collected by a collimating lens utilizing UV graded fused silica 1 meter, multimode sampling fiber of focal length ~0.4 m and spectral resolution $\frac{\lambda}{\Delta\lambda} \cong 9.2 \times 10^3$ with SMA connector and is transferred to a broadband spectrometer (LIBS2500+, Ocean Optics, Inc., USA).

Our LIBS2500+ was equipped with seven HR2000 high resolution (FWHM ≈ 0.1 nm) miniature spectrometers in 200-900 nm wavelength range. The integration time of the detector was set to be 2.1 ms and the time interval between the laser pulse and the start of spectrum recording (opening of the detector) was optimized and set 2 μs. To display the data in the form of spectrum, built-in software was used. In order to get the best signal-to-noise (S/N) ratio, each spectrum was recorded using a single laser shot and output data were averaged of twenty laser shots. Our LIBS system was calibrated by recording the well-known lines of neon and argon with an uncertainty of ~0.02 nm. The collected samples were first converted into pellets by putting 10 gm of each sample in a dye and pressing them by applying a load of 10 tons for 20 min in the dimensions ~4 × 6 cm and a thickness of ~2 cm and were pasted on target stand for ablation.

For analysis of Shilajit using ICP/OES, the samples were dissolved in concentrated HNO_3 and allowed to stand for 24 h. 10 mm of $HClO_4$ were added to the mixture heated in range (280 and 300°C). The liquid solution was analyzed by ICP-spectrometer (Optima 2100-DV; Dual View, PerkinElmer). The ICP spectrometer was calibrated to detect in organic elements only that is why Carbon was not detected using ICP analysis.

For the Infrared spectra, ~2 mg of the samples were blended with ≈100 mg KBr (potassium bromide) in a clean glass grinder and mortar and pressed together to convert them into pellets of thickness ~0.78 mm. The pellets were then treated using FTIR-spectrometer (Perkin-Elmer Spectrum GX).

Locations of samples collection

The Shilajit samples were collected at different locations from the Himalayan mountainous ranges in Pakistan. The collected Shilajit samples and their locations are tabulated in Table 1.

Results and Discussion

Studies of optical emission lines

In the first phase of this experimental work, we recorded the spectral emissions during cooling and recombination phase of plasma generated by Q-switched Nd:YAG laser at 1064 nm. The emission spectra of S1, S2, S3, S4, and S5 are shown in Figures 2-6. Careful study of the spectrum via the National Institute of Standards and Technology (NIST) spectral atomic data-base exposed the existence of neutral and ionic lines of aluminum, magnesium, calcium, iron, sulphur, and neutral carbon, potassium, lithium, silicon, sodium, oxygen, phosphorous, nickel, manganese, and hydrogen. The presence of hydrogen and oxygen line is due to water in air and the samples. Furthermore, the analysis was verified via the comparison of LIBS spectra of Shilajit with individual LIBS spectra of the pure elements.

Statistical analysis

Statistical investigation was carried out to study the consistency between the results of LIBS and ICP-spectroscopy. Student's t-test analysis was performed on the acquired spectrum. The analysis showed that the results were statistically consistent (t-test at $p>0.05$).

The agreement between the results using LIBS and standard technique was studied via performing the statistical correlation test on the acquired results (Figures 7-9).

The linear correlation coefficient was estimated using relation:

Samples locations	Samples names	Height from sea level (meters)
Skardu	S1	2438
Gilgit	S2	1500
Chitral	S3	1100
Chilas	S4	915
Muzaffarabad	S5	724

Table 1: Locations of collected Shilajit samples versus Height from sea level.

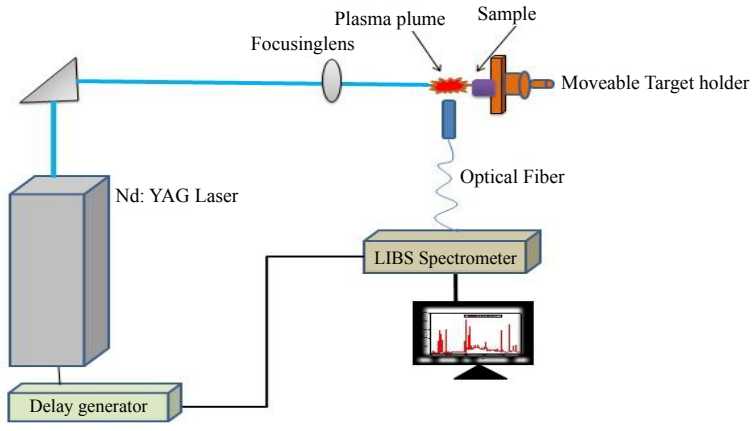


Figure 1: Schematic diagram of the experimental setup.

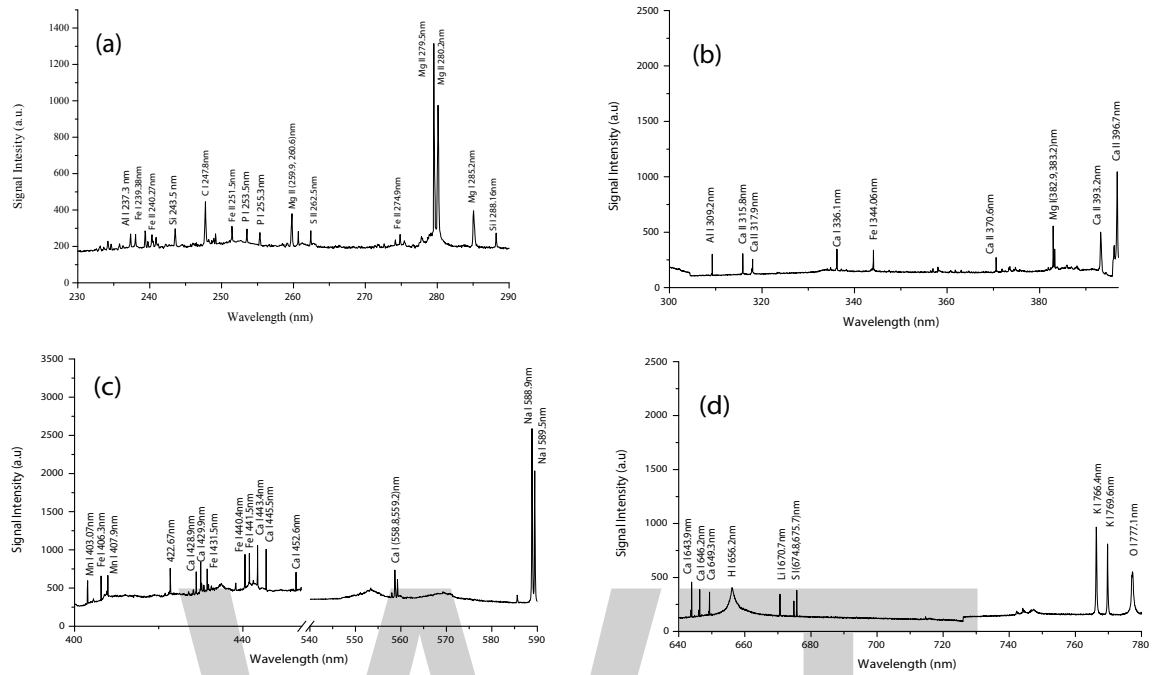


Figure 2: (a-d) LIBS emission spectrum of sample S1 covering spectral range 230-780 nm.

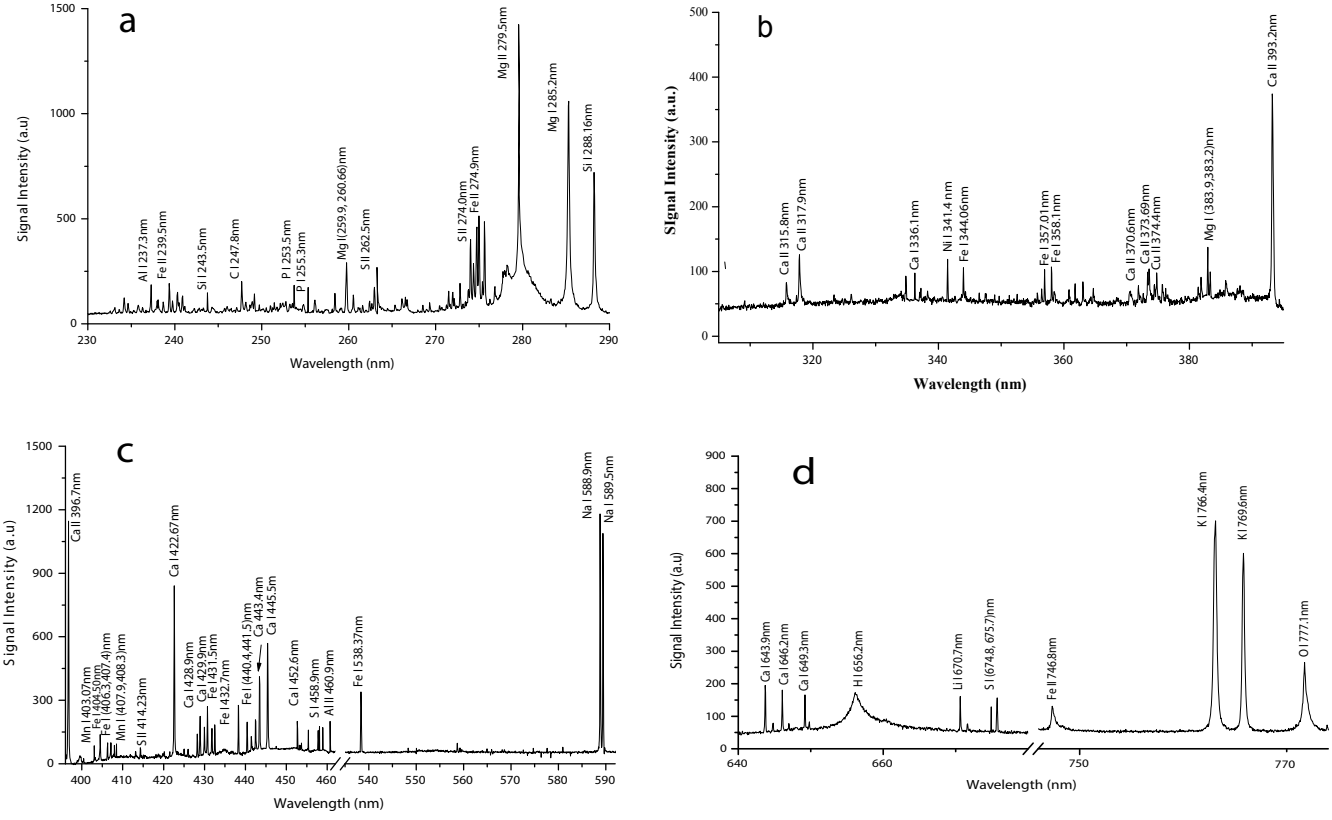


Figure 3: (a-d) LIBS emission spectrum of sample S2 covering spectral range 230-780 nm.

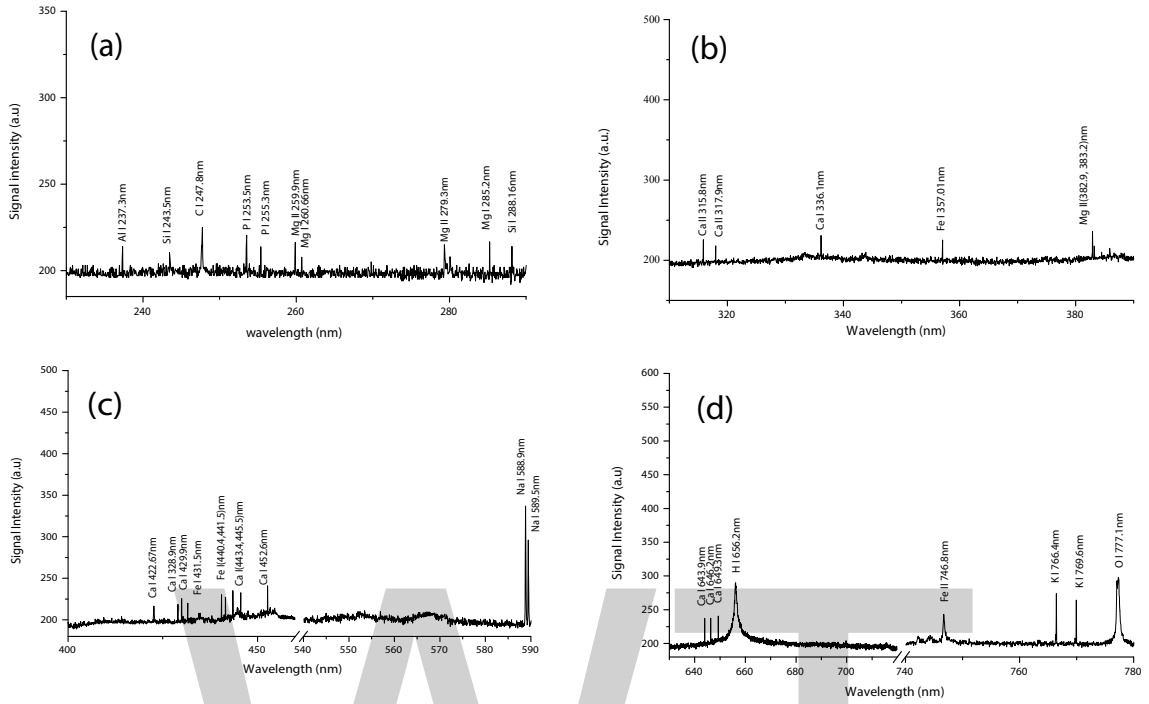


Figure 4: (a-d) LIBS emission spectrum of sample S3 covering spectral range 230-780 nm.

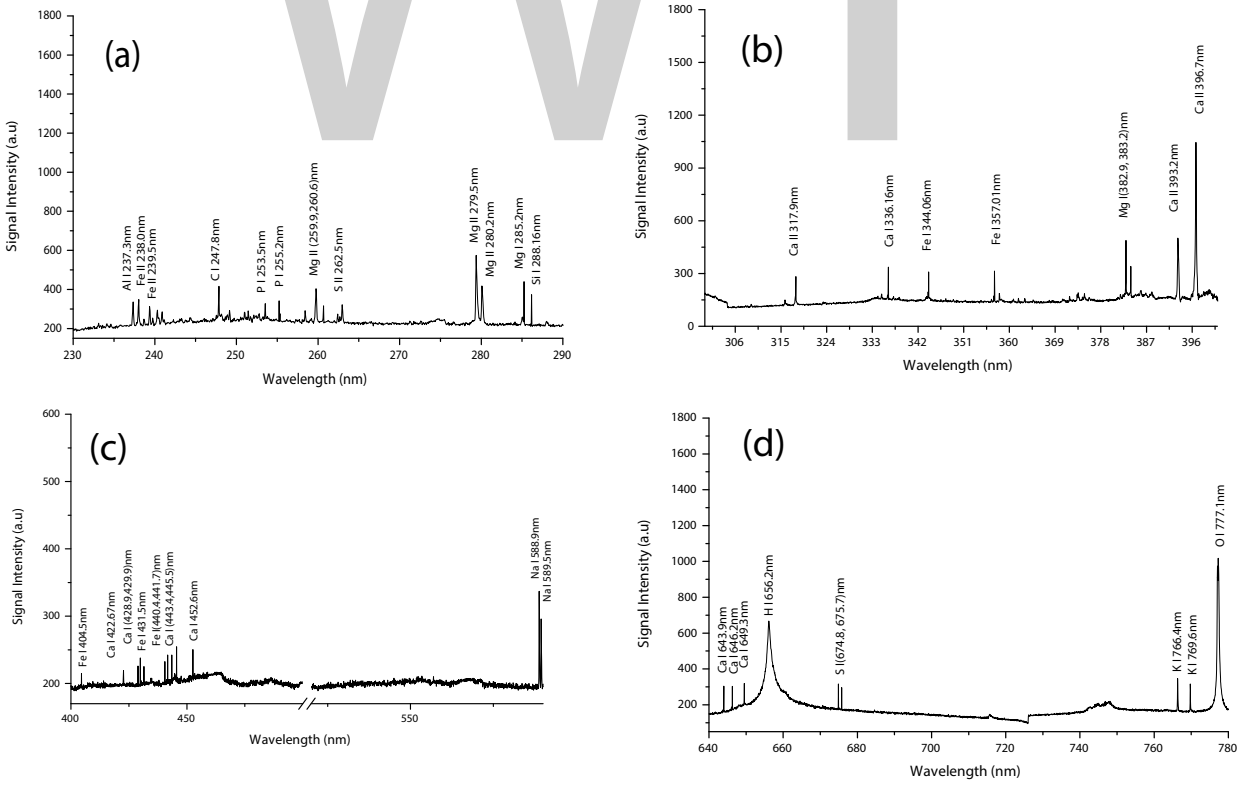


Figure 5: (a-d) LIBS emission spectrum of sample S4 covering spectral range 230-780 nm.

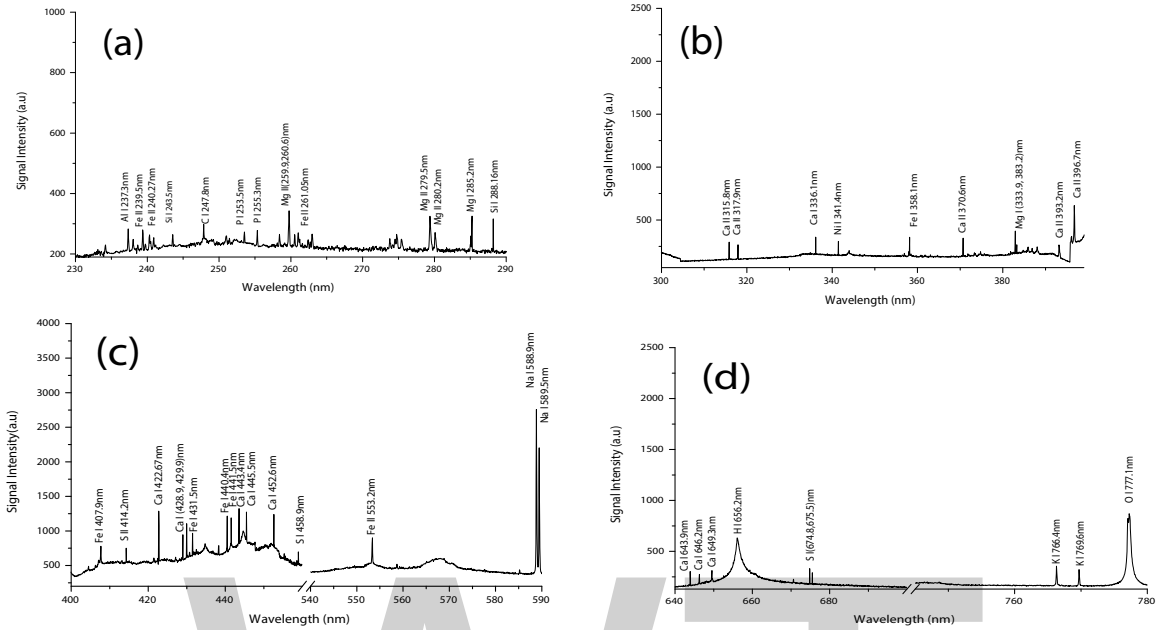


Figure 6: (a-d) LIBS emission spectrum of sample S5 covering spectral range 230-780 nm.

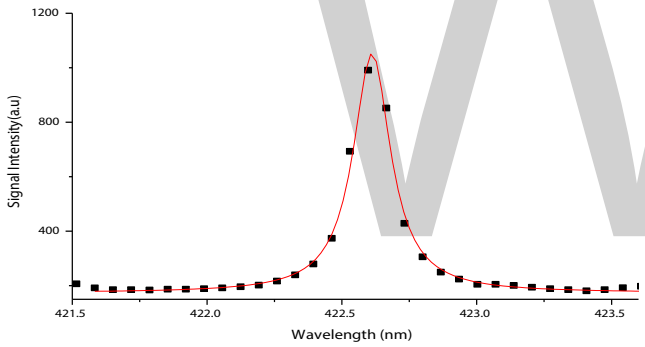


Figure 7: Lorentzian fitted profile to the experimentally data of Ca I spectral emission at 422.67 nm corresponding to (3p6 4s^2 $^1S_0$→3p^{6}4s4p$^1P^0_1$) transition.

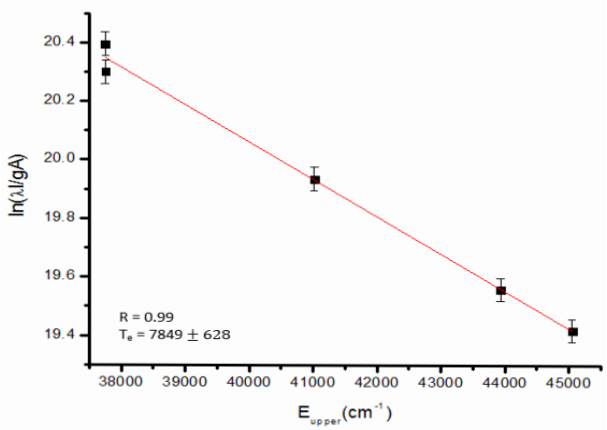


Figure 8: Typical Boltzmann plot for Ca I lines in S2 at 1064 nm having energy of 100 mJ/pulse.

$$r = \frac{\sum\left[\left(x_i - \bar{x}\right)\left(y_i - \bar{y}\right)\right]}{\left[\sum\left(x_i - \bar{x}\right)^2 \sum\left(y_i - \bar{y}\right)^2\right]^{\frac{1}{2}}} \quad (1)$$

Where, r is called correlation factor (-1 ≤ r ≤ 1).

Quantitative measurement

The atomic line's integrated intensity of a particular element can be used to measure its abundance in a sample [1,14,19,20]. To determine the relative concentration of different elements there in Shilajit, two calibration techniques were utilized. In the first technique, we selected the strongest line of each element whose integrated intensities were used to extract the relative concentrations in the samples. We used the following lines: Al at 237.3 nm, C at 247.8 nm, Ca at 422.67 nm, Fe at 440.4 nm, K at 766.4 nm, Li at 670.7 nm, Mg at 285.2 nm, Mn at 403.07 nm, Na at 588.9 nm, Ni at 341.4 nm, P at 253.5 nm, S at 675.7 nm and Si at 288.16 nm. The relative abundance (%-age) of elements measured by this formula in Shilajit samples are tabulated in Table 2 and plotted in Figure 9.

The second approach was calibration-free LIBS. This strategy does not require standard sample curves. The detail of this method has been presented in the literature [9,21]. This technique is based on supposition of Local Thermodynamic Equilibrium (LTE) and Optically Thin Plasma (OTP).

McWhirter criterion is one of the conditions for LTE is given [22,23]:

$$N_e\,(cm^{-3}) \geq 1.6 \times 10^{12}\, T^{1/2}(\Delta E)^3 \quad (2)$$

Where N_e is the electron number density, T gives the average plasma temperature, and ΔE gives the energy difference between upper and lower levels.

To verify the existence of LTE, the Ne was measured with the Stark-broadened line profile of the Ca-I line at 422.67 nm (Figure 3). The full width at half-maximum (FWHM) (Δλ1/2) of the line profile was 0.282(5) nm. The electron density was determined using the relation as:

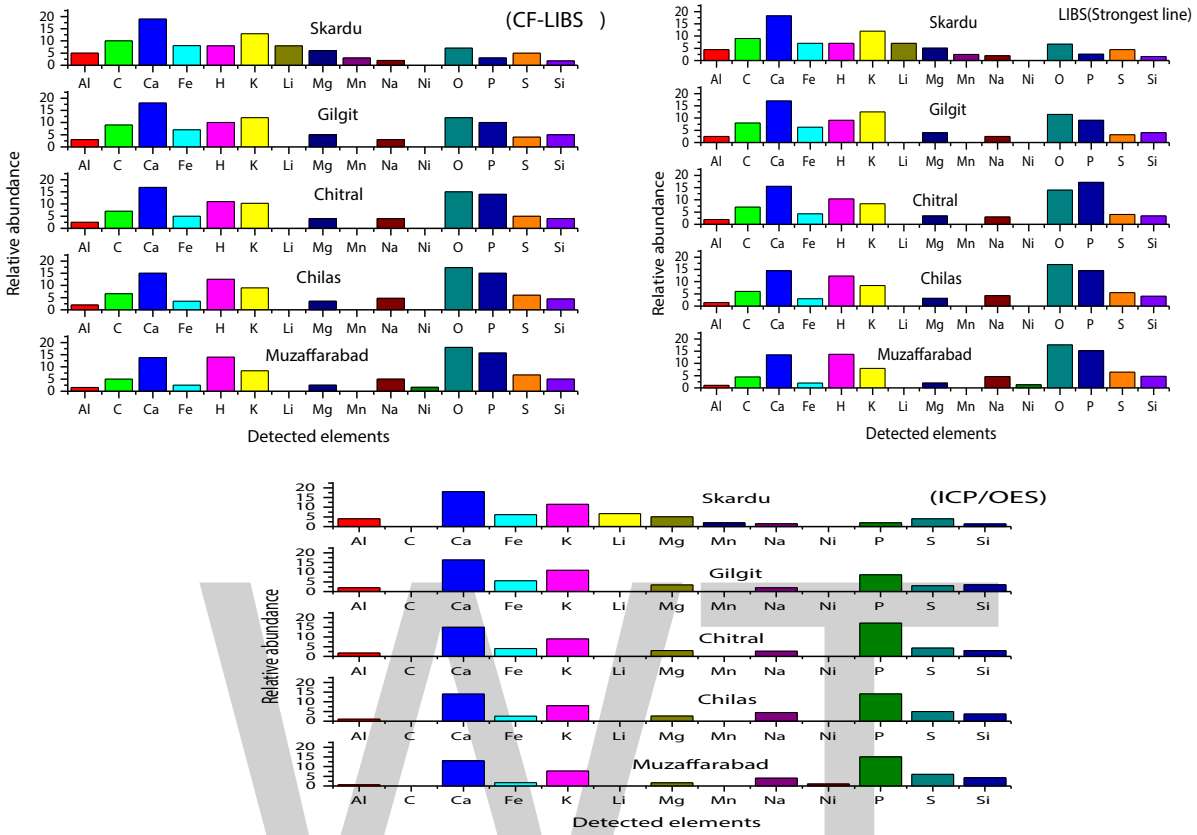


Figure 9: (a-c) Comparison between concentrations of elements in Shilajit using two LIBS calibration approaches and ICP/OES.

$$N_e = \frac{\Delta\lambda_{FWHM}}{2\omega} \times 10^6 \quad (3)$$

Where the term ω is called electron impact parameter and its value is taken from Griem [24]. For S1, the value of electron density was (5.8 ± 0.09 × 1017) and measured electron temperature via the Boltzmann plot method was 77.50 ± 620 K. After putting the plasma temperature and ΔE (eV) for the Ca I at 422.6 nm in equation (2), the resultant value was smaller than the value of electron density measured using equation (3). This verifies the LTE condition. The values electron densities for samples S2, S3, S4 and S5 are: N_e= 5.4 ± 0.09 × 1017, and N_e=5.1 ± 0.08 × 1017, N_e=5.0 ± 0.08 × 1017, Ne=5.2 ± 0.08 × 1017, respectively. The plasma temperatures for S2, S3, S4, and S5 are 7849 ± 627 K, 7700 ± 616 K, 7520 ± 670 K, and 7600 ± 530 K respectively. The measured electron densities (for sample-2, 3, 4, and 5) were also tested for McWhirter criteria and got verified.

The plasma is assumed to be optically thin, if the ratio of intensity of two non-interference emission lines from a species of same upper level and the ratio of their corresponding transition probabilities within the experimental uncertainty have the same values [25,26]. For this purpose, we compared the ratio of Ca I and Mg I lines to the ratio of their corresponding transition probabilities via NIST database. The ratio of Ca I at 428.94 nm/at 429.90 nm was 1.23 whereas; the ratio of their transition probability was 1.29. Similarly, the ratio of Mg I at 382.9 nm/at 383.2 nm was 1.33 and the ratio of corresponding transition probabilities was 1.39. This uniformity in the ratios between the intensity and their transition probabilities clearly declares the plasma is optically thin. Under LTE assumption, the line intensity of constituents present in target sample corresponding to a transition between levels E_k and E_i of the atomic species γ is

given by:

$$I_\lambda = C_\gamma A_{ki} \exp \frac{gk}{U_\gamma(T)} \frac{Ek}{kT} \quad (4)$$

where C_γ is the concentration of emitting element, G is called the experimental factor, E_k and g_k are the upper level's energy and statistical weights, T gives the plasma temperature (average), A_{ki} stand for transition probability, and Uγ gives the partition function of emitting elements and is given as:

$$U_\gamma(T) = \sum_k g_k e^{\left(\frac{-Ek}{kT}\right)} \quad (5)$$

Taking log of equation (4), and using the definition as below, where, $x = E_k$, $y = \ln \frac{G.C_a}{U_a(T)}$, $m = \frac{-1}{kT}$

$$q_\gamma = \ln \frac{G.C_\gamma}{U_\gamma(T)} \quad (6)$$

We obtained the linear relationship as:

$$y = mx + q_\gamma \quad (7)$$

This is a graphical illustration of Boltzmann plot. We measured the plasma temperature using its slope m. In Figure 8 a typical Boltzmann plot is shown for Ca-I emission lines whose linear fit displays a correlation coefficient close to unity.

Sample No.	Elements	CF-LIBS	LIBS(strongest line)	ICP/OES	Sample No.	Elements	CF-LIBS	LIBS(strongest line)	ICP/OES
Sample 1	Al	5	4.5	4	Sample 3	Mg	4	3.4	3
	C	10	9	ND		Mn	ND	ND	ND
	Ca	19	18.3	18		Na	4	3	2.7
	Fe	8.04	7	6.2		Ni	ND	ND	ND
	K	13	12	11.5		P	14	14.3	13.8
	Li	8	7	6.7		S	5.0.0	4	4.3
	Mg	6	5.04	5.04		Si	5	3.5	3
	Mn	3	2.5	2	Sample 4	Al	2	1.5	1
	Na	2	1.98	1.5		C	6.6	6	ND
	Ni	ND	ND	ND		Ca	15	14.43	14
	P	3	2.54	2		Fe	3.5	3	2.6
	S	5	4.42	4		K	9	8.4	8
	Si	1.8	1.6	1.4		Li	ND	ND	ND
Sample 2	Al	3	2.53	2		Mg	3.5	3.2	2.7
	C	9	8	ND		Mn	ND	ND	ND
	Ca	18	17	16.3		Na	4.7	4.3	4.5
	Fe	7	6.3	5.6		Ni	ND	ND	ND
	K	12	12.5	11		P	15	14.5	14.1
	Li	ND	ND	ND		S	6	5.5	5
	Mg	5	4	3.4		Si	4.5	4	3.7
	Mn	ND	ND	ND	Sample 5	Al	1.57	1	0.57
	Na	3	2.5	2		C	5	4.5	ND
	Ni	ND	ND	ND		Ca	13.78	13.5	13
	P	10	9.1	11		Fe	2.53	2	1.7
	S	4	3.2	8.7		K	8.42	8	7.7
	Si	5	4	3		Li	ND	ND	ND
Sample 3	Al	2.5	2	1.7		Mg	2.5	2	1.59
	C	7	7	ND		Mn	ND	ND	ND
	Ca	16.8	15.5	15		Na	5	4.6	4
	Fe	5	4.3	4		Ni	1.66	1.3	1
	K	10.3	8.4	9		P	15.7	15.2	15
	Li	ND	ND	ND		S	6.7	6.4	6
						Si	5	4.7	4.2

ND: Not Detected

Table 2: Table showing the relative abundance of detected elements using LIBS and ICP/OES.

After calculating partition function for each known elements (using Equation 5), and the plasma temperature, the experimental factor G may was estimated via normalizing the sum of the species abundances:

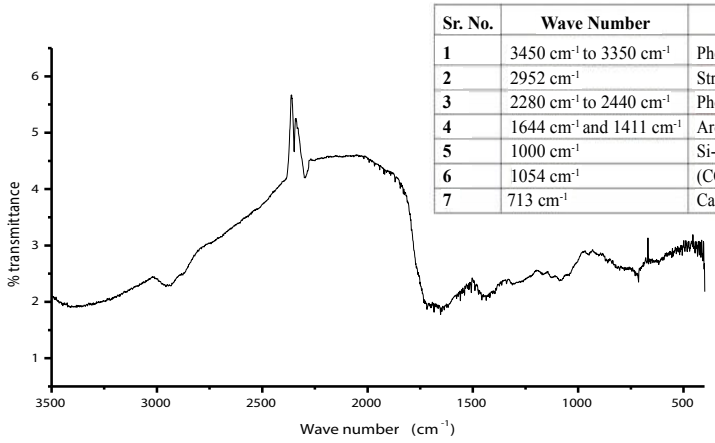
$$\sum_{\gamma} C_{\gamma} = \frac{1}{F} \sum_{ó} U_{ó}(T) e^{(q_{\sigma})} = 1 \quad (8)$$

Hence

$$F = \sum_{\gamma} U_{\gamma}(T) e^{(q_{\gamma})} \quad (9)$$

qγ is the intercept of the straight line of the Boltzmann plot (Equation 7) which is proportional to the logarithm of the concentration of species times by the factor G. After the determination of partition function Uγ(T), the experimental factor G, and the intercept qγ (through Boltzmann plot method), we used Equation 6 to estimate concentration Cγ of every elements. For the relative concentration, the concentration of each element was divided by the total concentration of all elements. The relative concentration or abundance of the species acquired through calibration-free LIBS is exposed in Figure 5a-5c.

Mutual comparison of relative concentration using LIBS and ICP/OES of the detected species in Shilajit samples was exposed in Figure 9. Carbon and calcium has increasing tendency, which may be a result of constant carbon inputs and decrease in rate of carbon loss or may be due to Carbon sequestration in soil [27]. The phosphorous content diminishes with the altitude [28]. Similar to carbon and calcium, aluminum also shows expanding pattern with height [29]. Manganese and iron also increase with altitude. Additionally, in some regions Li, Mn, and Ni were not detected because of low concentration and were below the detectable range of our experimental setup. Furthermore, silicon was available in low amount in all the gathered samples. As the Shilajit from more height contains more aluminum, calcium, magnesium and potassium, therefore Shilajit collected from the more heighted location may be useful for keeping strong muscle, strong bones, and maintaining blood pressure normal as well as for heart rhythm problems. It may also be helpful in reducing the problems of kidney stone [30-33]. On the other hand Shilajit from lower altitude may be helpful in keeping balance hormonal and digestion process [34,35] helpful in the formation of protein. In the same way, it may use to contract muscle and maintain the body's fluid and electrolyte balance [31], due to abundant amount of Phosphorous present in the Shilajit acquired from lower altitudes. Carbon was not detected using ICP-analysis in all of the collected samples because ICP spectrometer was able to detect in-organic metals only.



Sr. No.	Wave Number	Functional Group
1	3450 cm^{-1} to 3350 cm^{-1}	Phenolic functional group
2	2952 cm^{-1}	Stretching of aliphatic carbon and hydrogen
3	2280 cm^{-1} to 2440 cm^{-1}	Phosphine (P-H)
4	1644 cm^{-1} and 1411 cm^{-1}	Aromatic carbon-carbon double bond
5	1000 cm^{-1}	Si-OR
6	1054 cm^{-1}	(COOH) carboxyl groups
7	713 cm^{-1}	Calcite

Figure 10: Representative FTIR spectrum of sample S2.

Fourier Transform Infrared Spectroscopy (FTIR)

The FTIR spectroscopy was performed in base line range from 3500-500 cm^{-1} for of all the acquired samples. The representative FTIR spectrum of S2 was exposed in Figure 10. The Shilajit samples were characterized by relatively few broad bands. The spectrum reveals a broadband region of 3450 cm^{-1} to 3350 cm^{-1} due to the vibration of hydrogen bonded hydroxyl of the phenolic functional group. The appearance of the band at 2952 cm^{-1} may be credited to the stretching of aliphatic carbon and hydrogen. The Peak at 1000 cm^{-1} may be due to Si-OR. The range 2280 cm^{-1} to 2440 cm^{-1} corresponds to phosphene. Weak bands in the region of 1644 cm^{-1} and 1411 cm^{-1} may be attributed to the aromatic carbon-carbon double bond, H-bonded C=O and O-H bending vibrations of COOH (carboxylic) and OH (alcohols) respectively.

Diminishing peak tendency at 1054 cm^{-1} may be indorsed to the OH deformations of (COOH) carboxyl groups. Similarly, the Peak at 713 cm^{-1} may be ascribed to Calcite. The vagueness of the peaks suggests the presence of lower molecular weight compounds due to the chemical breakdown of animal and plant remains under specific geothermal conditions leading to the generation of lignin, protein, and polyphenols along with lipids. All these findings were strongly supported by the already documented literature. Similar is the case for other Shilajit samples. In the present work, ICP/OES was used as a standard method. Figure 5 shows that the relative concentration acquired via the integrated line intensity of the strongest peak for every element gives the outcome close to that of the ICP/OES study with exceptions of carbon. These experimental findings were verified using statistical correlation test.

Conclusion

Spectroscopic analysis of the collected Shilajit samples was performed using LIBS. The elements like Al, C, Ca, Fe, K, Li, Mg, Mn, Na, Ni, P, S and Si was detected. Our findings suggest the altitudinal variations in Physico-chemical properties of Shilajit. Elements like Al, C, Ca, Mg, Mn and Fe showed an increasing trend with altitude whereas, Na, Ni, P, S have decreasing tendency with respect to the height from ground level. The elements Li, Mn, and Ni were below the detectable range of our experimental setup for some locations. The altitudinal variations of observed elements were due to atmospheric and soil properties. To identify different functional groups, we used FTIR-Spectroscopy. These measurements confirm that the relative concentration using the strongest line of an element provide values were in good comparison to the results obtained with ICP/OES that yields a high correlation factor. The results show that LIBS is fast and reliable method for food classification and detection of food contents.

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Nutritional Status of Children in Slums of Dhaka, Bangladesh

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Abstract

The nutritional status of children in slums of Dhaka is a sensitive indicator of health, economy and the sustainable development of Bangladesh. The research highlights the consequence of anthropometric measurements and nutritional status related other issues are taken of 102 children. The survey with a structured questionnaire was conducted by the researcher during 15th April 2015 to 6th June, 2015 among 102 children aged between 2 to 12 years from Mirpur, Kuril and Kamalapur slums (N=102) in Dhaka city. To determine hypothesis statistical data analysis QtiPlot and MS Excel software tools were used and hypothesis was tested. Bi-variant and analyses were used to determine the nutritional status of children and identify the relationship of common factors with the nutritional status of children. The research presents a number of results, 33.5% of the children age between 2 to 12 years are suffering from malnutrition. According to the BMI results, 32% of the children have undernourished problem. The socio-economic status of the children is pitiable. The children families mean monthly household incomes are affecting their health and nutritional status. The study reveals that 76.4% of the children are suffering from diseases for last one month because of their sanitation and hygiene status. The research also highlights the nutritional status of the children influenced by their parents awareness, socio-economic and demographic condition, nutrient intake, breastfeeding practice, good hygiene practice, sanitation system. Malnutrition along with high morbidity rates, this action may occur the children from their future unexpected problem during motherhood and hamper cognitive development. Nutritional education programs need to be implemented to improve the nutritional status. The present study generates information which indicates that the nutritional status of the children should not be overlooked and suitable approaches designed to improve their nutrition should be considered and studies elaborately in the future.

Keywords: Slums; Malnutrition; Nutritional status; Food intake; Hygiene and sanitation

Introduction

Bangladesh is the ninth most populous countries in the world [1], 1,203 persons per sq. km are living in Bangladesh [2] and Dhaka the capital of Bangladesh, one of the most densely populated cities in the world [3]. The population in slums of Dhaka city is increasing at an alarming rate due to migration by the rural poor. Slums are the spatial symptoms of urban poverty, social exclusion, and improper government policies [4]. Almost 28% people of Dhaka city are living in the slums under very miserable condition [5] and most of them are undernourished, illiterate and do not have knowledge about the nutritional value of foods [6]. Due to poor sanitation and congested living with 4 to 5 people, they often suffer from diseases like diarrhea, dysentery, typhoid, dengue and pneumonia [7]. Specially, children who are naturally innocent, vulnerable, and dependent often suffering from malnutrition [8]. The nutritional status of these children is an alert sign of the country's health, sustainable nutrition and economy. Malnutrition of children is a serious problem in slums of Dhaka [9]. Malnutrition is one kind of situation in which long-lasting lack of one or more nutrients retards physical development or causes specific clinical disorders, e.g., low birth weight, wasting, stunting, underweight, vitamin A deficiency, iodine deficiency disorder, iron deficiency anemia, etc., [10]. Bangladesh is one of the countries with the highest rate of children malnutrition [11] and up to 40% of children under the age of 5 years are suffering from chronic malnutrition [12] and malnutrition rate of children in slums is even worse [13]. Protein-energy malnutrition, iron deficiency anemia, iodine deficiency disorders, and vitamin A deficiency are common for children in the slums of Dhaka city [14] and the main reasons for that their poor social, economic and demographic conditions, including family income, assets, morbidity, employment, total household expenditure, mother's education, social networks, hygiene and sanitation and consumption knowledge [7,15]. One of the ICDDRB's Centre for Nutrition & Food Security baseline survey in Bangladesh showed that under nutrition is high among young children, 41% of children were found stunted, 33% underweight and 11% wasting [16].

Research aim and objectives

The research carried out to assess the nutritional status of under the age of 2 to 12 years children in slums of Dhaka city.

The associated major objectives include:

- To find out the nutritional status of the children through anthropometric measurements and dietary assessment.
- To find out the demographics and socio-economical characteristic of the study children.
- To determine the health, and sanitation condition of the children.

Materials and Methods

Research site

In this research, Mirpur slum of Mirpur Thana, Kamalapur slum of

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Motijheel Thana and Kuril slum of Badda Thana in Dhaka district have been chosen for the survey.

Research design a survey was carried out among 102 children of Dhaka slums age between 2 to 12 years during April to June, 2015 with format permission from the local authority and by taking informal consent. It was a cross-sectional survey and divided into three clusters (Mirpur, Kuril and Kamalapur slums) in Dhaka city. Dietary data, socioeconomic, demographic, disease variables and anthropometric data - height, weight and BMI was collected by using a structural questionnaire and nutritional status assessed. The research has been conducted in two steps.

Firstly, the necessary data has been extracted from anthropometric cross-sectional health survey and each child gave a sample of food frequency.

Secondly, the extracted data has been assessed and compared with certain standards by analyzing specific contents.

Data collection

Data collection is the process of gathering information. The data collection process of this study was a primary research (field research). Total 102 children (respondents) have been selected of three different slums in Dhaka city and samples had been taken from these three clusters (slums) by using the questionnaire and made the interviews. Random sampling technique was used for data collection. Random Sampling is a probability sampling method, starting point is determined randomly and from then on the systematically each nth element is drawn [17]. The children's own mother language Bengali had been used during data collection.

Data analysis

For data analysis an important first step is summarizes and display of the data [18]. In the current research, data were edited. Editing involves carefully checking survey data for completeness, legibility, consistency, and accuracy. The collected data were evaluated, categorized, grouped and interpreted aiming at the objectives of the study. Then, the relevant data processed and analyzed by using statistical software QtiPlot and Microsoft excel. In quantitative research, tables and graphs are used to display data and convey meaning in the analysis [19].

Table 1 illustrates a total 102 children have studied. Of the participants 32.35% are aged 6 to 9 years, 48.04% are aged between 2 to 5 years and the remaining 19.61% are between 10 to 12 years old.

Table 2 shows that total 31.8% children BMI are underweight (<14.5). On the other hand, 66.7% children's BMI normal (14.5-21.5) and 2% are overweight.

Figure 1 show that 62% of the children's family household income 8,000 to 12,000+BDT (Bangladeshi Taka), 6% of the children's household income is 3,000 to 5,000 BDT and 32% is 5,000 to 8,000 BDT. But the incomes of these three groups are not enough for their living, accommodation, foods and others.

Table 3 shows that 6% of the children monthly household incomes are between Tk 3,000-5,000 and undernourished (<14.5) 83%. On the other hand, 32% of the children monthly household incomes are between Tk 5,000-8,000 and undernourished almost 40% and 62% of the children monthly household incomes are between Tk 8,000-12,000 and undernourished almost 22%.

Table 4 shows that the relationship amongst the nutrient intake and per capita income of the children. The children are divided into three groups based upon the household per capita income. Children from lower income families calorie, protein and iron intake which are seen lower than the children from higher income families. Mean energy, protein and iron intake are highest 1102.4859 kcal, 16.55 g, 5.70 mg respectively, for the children who are from families of highest per capita income between Tk. 8,000-12,000/month.

Table 5 illustrates that the mean height of the children (cm) are 109.72 ± 5.6, weight (kg) was 15.83 ± 9.4 and Mean Body Mass Index (BMI) (kg/m2) are 15.74 ± 3.5. Mean BMI is 15.74 which is acceptable but not enough because 32% children are under nourished.

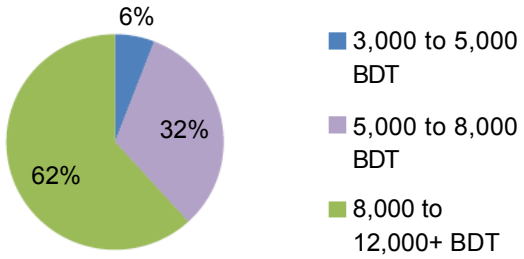
Age (year)	Frequency (N)	Percent (%)
2-5	49	48.04
6-9	33	32.35
10-12	20	19.61
Total	102	100

Table 1: Distribution of the children's by their age group (N=102).

Age Group (years)	N (%)	Under nourished (<14.5) n (%)	BMI Normal (14.5-21.5) n(%)	Overweight (21.5+) n (%)
2-3	49 (48.039)	18 (36.72)	29 (59.20)	02 (4.08)
6-9	33 (32-352)	09 (27.27)	24 (72.73)	00 (00)
10-12	20 (19.607)	05 (25.00)	15 (75.00)	00 (00)
Total	102 (%)	32 (31.8%)	36 (66.7%)	02 (1.97%)

Source: (author's own construction and calculation according to own data source with compare to WHO children's BMI charts).

Table 2: Distribution of the children's by age group with related to Body Mass Index (BMI) (aged 2 to 12 years) (N=102).



Source: (author's own construction and calculation according to own data source).

Figure 1: Distribution of the children's by monthly household income (N=102).

Per capita income (BDT)	N (%)	n (%)	BMI
3,000-5,000	6 (%)	5 (83.33)	Under nourished (< 14.5)
		1 (16.67)	Normal (14.5-21.5)
		00 (00)	Overweight (21.5+)
5000-8,000	33 (32%)	13 (39.4)	Under nourished (< 14.5)
		20 (60.6)	Normal (14.5- 21.5)
		00 (00)	Overweight(21.5+)
8,000-12,000	63 (62%)	14 (22.2)	Under nourished (< 14.5)
		47 (74.6)	Normal (14.5- 21.5)
		02 (3.2)	Overweight (21.5+)
Total	102 (100)	102	BMI (14.5 to 21.5+)

Source: (author's own construction and calculation according to the own data source).

Table 3: Distribution of the children's by monthly household income with related to nutritional status/BMI (N=102).

No. of Children	Mean calorie intake (kcal)	Mean Protein intake (g)	Mean iron intake (mg)
6	986.66	13.92	5.002
33	1029.75	16.87	6.106
63	1102.48	18.84	6.018
102	1039.4	16.55	5.7

Source: (author's own construction; the data was calculated on the basis of WHO/FAO technical committee dietary guidelines for children's data).

Table 4: Relationship between per capita income and nutrient intake (N=102).

Characteristic	Minimum	Maximum	Mean	St. D.
Height (cm)	81.28	147.32	109.72	± 5.6
Weight (kg)	11	30	15.83	± 9.4
BMI (kg/m2	11.44	18.16	15.74	± 3.5

Source: (author's own construction; own primary data source).

Table 5: Anthropometric characteristic of the children (N= 102) (aged 2 to 12 years).

Food Sources	Mean food intake (g/d)	Percentage (%)
Cereals	131.31	45.19
Pulses	14.91	5.13
Green Leafy Vegetables	22.1	7.61
Non Leafy Vegatables	24.26	8.35
Fruits	12.45	4.29
Milk, fish & egg	30.68	20.88
Milk & milk products	24.87	8.55
Total	290.58	100

Source: (author's own construction and calculation; according to own data).

Table 6: Mean per capita food intake per day (N=102).

Table 6 shows that total children mean food intake 290.58 gm per day. The contribution from cereals is 131.31 gm which is 45.19% of total food intake per day almost half of the total food intake. Food consumption from animal sources such as meat, fish and egg are 60.68 gm which is 20.88% of total food intake and animal dairy & dairy products are 24.87 g per day which is 8.55% of total food intake per day per children and others food intake respectively.

Table 7 shows that the mean calorie intake is 1039.40 kcal, protein intake are 16.55 g/d. The mean CHO, fat, vit-C, and iron and calcium intake are found 139.53 g, 14.11g, 22.72 mg, 5.70 mg and 407.75 mg respectively/day.

Table 8 illustrates that the mean daily energy requirement on the basis of their age group of the studied children are 1316.66 kcal and most mean energy balance deference is -387.52 kcal of the age group 10 to 12 years of children but age group 2 to 5 years old children mean energy balance deference looking-191.74.

Table 9 shows that per capita energy and nutrient intake and mean percentage fulfillment of the requirement. The table shows gross inadequacy in terms of % fulfillment of the recommended allowance for energy, protein, iron, fat, CHO, vitamin-C and calcium and different age group of children with different amount of nutrients requirement and intake. The percentage of nutrients fulfillment/day is lower than RDA and it came from seven individual nutrients of foods e.g. energy (kcal) 76%, protein 68%, fat 52%, iron 69%, calcium 43% and CHO 76% mean fulfillment per day. Vitamin-C intake is 82% and higher percentage than others nutrient groups.

Table 10 shows that the relation between BMI and nutrient intake. 68 children has normal BMI and they consumed 1040 ± 319 kcal energy, 18 ± 10 g protein, 14 ± 8.5 g fat and 175 ± 46 g CHO. On the other hand, 32 children are undernourished and their BMI are below 14.5. They consumed 874 ± 325 kcal energy, 14.5 ± 9 g protein, 12.5 ± 9 g fat and 113 ± 58 g CHO.

Table 11 reveals that current energy intake reduced by 60.60 kcal as compared with icddr'b-2010 national survey. But compared to the WHO-2000 and WFP-2008 survey with the current research, the energy intake is slightly increased. The energy intake ratio of the children is fluctuated but not big margin the table shown. On the other hand, the current, Bangladesh study shows that protein intake

Nutrients	Mean per capita intake
Cal. intake (kcal)	1039.4
Protein (g)	16.55
Fat from foods (g)	14.11
Carbohydrate (g)	139.53
Vitamin C (mg)	22.72
Iron (mg)	5.7
Calcium (mg)	407.75

Source: (author's own construction; data calculation on the basis of FAO-Infants, children, and adolescents energy, protein and micronutrients requirement data).

Table 7: Mean per capita nutrient intake by food sources (N=102).

Age group (years)	No. of children N (%)	Mean energy requirement/ day (kcal)	Mean energy intake /day (kcal)	Mean energy balance/d ay (kcal)
2-5	49 (48.039)	1150	958.26	-191.74
6-9	33 (32.352)	1300	1047.4	-252.55
10-12	20 (19.607)	1500	1112.4	-387.52

Source: (author's own construction; the data was calculation on the basis of WHO/FAO data).

Table 8: Distribution of the children's by their mean energy intake per day (N=102).

Nutrients	Age group (years)	Intake	Requirement	% of Fulfilment	Mean % of Fulfilment
Energy (kcal)	2-5	958.26	1200	79%	76%
	6-9	1047.4	1350	77%	
	10-12	1112.4	1500	74%	
Protein (g)	2-5	13.43	19	70%	68%
	6-9	17.17	24	71%	
	10-12	19.04	29	65%	
Fat from food (g)	2-5	13.02	20	65%	52%
	6-9	14.55	30	49%	
	10-12	14.78	35	42%	
CHO (g)	2-5	115.70	158.1	73%	76%
	6-9	132.07	178.7	73%	
	10-12	170.84	206.2	82%	
Vit-C (mg)	2-5	13.55	15	90%	82%
	6-9	21.40	25	85%	
	10-12	33.21	45	73%	
Iron (mg)	2-5	5.002	07	71%	69%
	6-9	6.106	10	61%	
	10-12	6.018	08	76%	
Calcium (mg)	2-5	333.50	700	48%	43
	6-9	415.09	1000	42%	
	10-12	474.41	1200	40%	

Source: (author's own construction and calculation according to own data source with compared to WHO/FAO data) Here age group 2-5 years: n=49; 6-9 years: n=33 and 10-12 years: n=20.

Table 9: Per capita energy and nutrient intake and mean percentage fulfillment of the requirement (N=102).

BMI	Num. of children	Energy (kcal)	Protein (gm)	Fat (gm)	CHO (gm)
<14.5	32	874 ± 325	14.5 ± 9	12.5 ± 9	113 ± 58
14.5-21.5	68	1040 ± 319	18 ± 10	14 ±8.5	175 ± 46
21.5+	2	1204 ± 41	17 ± 1	16 ± 1.5	131 ± 11

Source: (author's own construction and calculation according to own data source).

Table 10: Distribution of subjects by Body Mass Index and nutrient intake (N=102).

Nutrients	WHO-2000	WFP-2008	ICDDR'B-2010	Current study
Energy(kcal)	1020	1007	1100	1039.4
Protein(g)	26	22	19.5	16.6

Source: (author's own construction; comparison with WHO, WFP, ICDDR'B and current study's data).

Table 11: Comparison of per capita energy and protein intake of the children's with WFP-2008, WHO-2000 and icddr'b-2010.

decreased gradually and it is 16.6 g/day but 15 years before it was 26 g/day.

Figure 2 illustrates that total 78 children are suffering from diseases from last one month which is 76.4% of the total children.

Figure 3 Indicates that 31% children suffering from fever, 20% diarrhea, 23% cold & cough and these diseases are common issues for children in slums. The figure also shows that 7%, 12% and 7% of the children suffering from pneumonia, skin disease and jaundice respectively.

Table 12 indicates that there is a relationship between the morbidity patterns (last one month) of the children related with BMI. 91% of undernourished children suffering from any sort of diseases. On the other hand, the study found that, 72% of the normal children are also suffering from any sort of diseases. The underweight children morbidity rate is higher than the others BMI group children though. But there is no big margin between BMI and the morbidity pattern of the children.

Table 13 reveals that the types of water children's and their family used for drinking and for household tasks. Here children's family, N=54; Total children's 102, so 102/54=1.88 this value are average children per house. Highest 41% family are using supply water for drinking and another major source is tube well water which are 28% but 31% family used supply & tube well water both for drinking. On the other hand, 30% family is using supply water for household works and 22% family is using tube well water. 37% family is using supply & tube well water both for household tasks. But one of the concerning issues is that 11% of family are using cannel/river water which is so much dirty and it is not permitted for using.

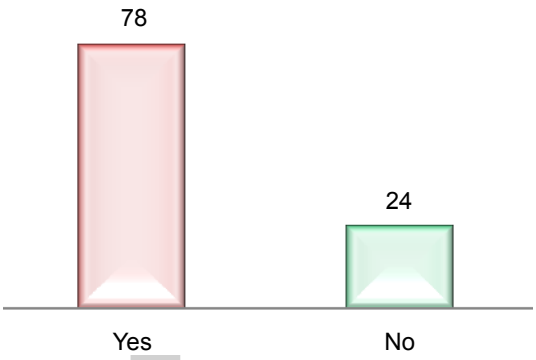
Table 14 shows that the types of human and household waste disposal of the children. 50% family are disposing their human waste near outside the house, 35% of the children with family disposing human waste far away outside the house and 15% of the children and their family disposing human waste near outside the house & far away outside the house both. On the other hand, 59% of the family is disposing their household waste near outside the house, 26% are disposing household waste far away outside the house and 13% are disposing household waste near outside the house & far away outside the house both. Only 2% of the children's family found they put household waste inside the house.

Figure 4 illustrates that the types of latrine children using for human waste disposal. Here the figure shows that 59% of the children are using non-sanitary latrine and 33% are using sanitary latrine. On the other hand, 8% children use sewer (open drain).

Table 15 indicates that the relation of the health issues like morbidity pattern with using water, human waste disposal and household waste disposal status of the children. 75% of the children are suffering from disease and it is related with the types of drinking water. On the other hand, 79% of the children are suffering from disease with related to household used water. Similarly, 75% and 80% children's disease also related with human waste disposal and household waste disposal respectively.

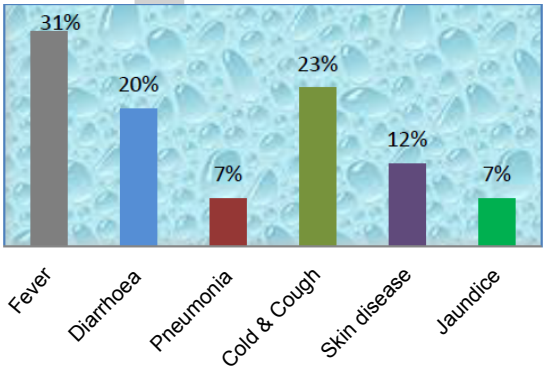
Discussion

The nutritional status of the slums children is a sensitive indicator of the country's health, sustainable nutrition and economy. This



Source: (author's own construction and calculation according to own data source).

Figure 2: Distribution of morbidity pattern among the children of last one month (N=102).



Source: (author's own construction and calculation according to own data source).

Figure 3: Distribution of the children's by disease (N=78) (according to figure 1).

BMI	No of Children's (N)	Suffered from diseases	Not sufferes from disease	% suffered from disease
Under nourished	32	29	3	91
Normal	68	49	19	72
Over weight	2	0	2	0
Total	102	78	24	76

Source: (author's own construction and calculation according to own data source).

Table 12: Relation between the morbidity patterns (last one month) of the children's related with BMI (N=102).

Sources of Water		No. of Children's family (N=54)	Percentages (%)
For drinking	Supply water	22	41
	tube well water	15	28
	Cannel/river water	0	0
	Supply & Tube well water both	17	31
For household works	supply water	16	30
	tube well water	12	22
	Cannel/river water	6	11
	Supply & Tube well water both	20	37

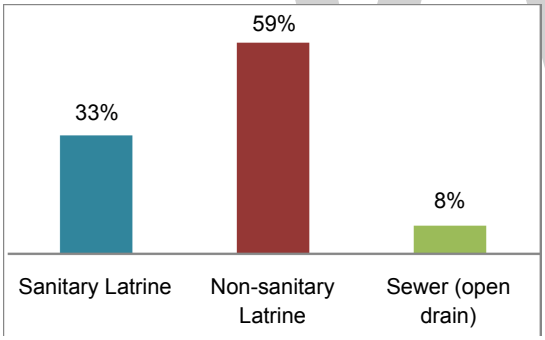
Source: (author's own construction and calculation according to own data source) Here children's family, N=54; Total children 102, so 102/54=1.88 this values are average children's per house.

Table 13: Distribution of the source of water using the children's family per day (total children's family 54 and number of children's 102) (N=54).

Sources of water		No. of children's family (N=54)	Percentages (%)
For household waste disposal	Inside the house	3	2
	Near ourside the hourse	32	59
	Far away outside the house	14	26
	Near outside the hourse & Far away outside the house	7	13

Source: (author's own construction and calculation according to own data source).

Table 14: Distribution of the human waste and household waste disposal status of the children's family (total children's family 54 and number of children's 102) (N=54).



Source: (author's own construction and calculation according to own data source).

Figure 4: Distribution of the types of latrine children's have used for human waste disposal (N=102).

present study reveals that children aged between 2-5 years are found 48% (Table 1), almost half of the total study children which is very sensitive age group of the children's whole life. Because, 2-5 years of children are suffering from malnutrition more (36.72%) than the other two groups of children in slums of Dhaka (see Table 2). According to the WFP-2009, malnourished children are birth to 5 years old at high risk of morbidity and mortality rate. Current research reveals that cereal food groups, especially rice has eaten daily (20-21 times/week) by 96.07% of the children. Therefore, malnutrition is a serious problem of children in slums of Dhaka [9]. According to the Child Nutrition Survey of Bangladesh 1990- 2005, during 1990 to 2005, the prevalence of underweight was reduced by 25.2 percentage points which was from 70.9% to 45.7% and stunting by 29.1 percentage points from 68.3% to 39.2%. It was a significant reduction and the survey expected that Bangladesh achieves the target of reducing underweight prevalence to 32.9% in 2015. Another survey conducted by Bangladesh Bureau of Statistics (BBS) (MICS 2012-13) and according the BBS survey and WHO 2005 growth reference standards, almost 33% of children aged in between birth to 6 years were undernourished in Bangladesh. The World Health Organization illustrated that the undernourished rate was "very high", and the prevalence of wasting point out a "critical problem" in Bangladesh [20]. In Dhaka division the nutritional status of children had the lowest prevalence of wasting which was 9.7% but when it made ratio with slums children it was more than 9.7% (WHO, 2005). The current study indicates that the mean Body Mass Index (BMI) (kg/m2) of the children is 15.74 ± 3.5. Mean BMI are 15.74 which is acceptable but not enough because 32% of the children's BMI are under nourished (<14.5) (see Table 2). The study also shows that 33.5% of the children under the age between 2 to 12 years are suffering from malnutrition because of the total percentages of nutrients fulfillment per day is lower than Recommended Dietary Allowances (RDA) set by the relevant national and international body including ICDDR'B, Bangladesh, National Nutrition Council (NNC), Bangladesh, WHO and FAO for children. This prevalence of percentage comes from seven individual essential nutrients of food such as energy 76%, protein 68%, fat 52%, iron 69%, calcium 43% and CHO 76% mean fulfillment per day (see Table 9).

On the other hand, the present study illustrates that the socio-economic status of the slums children in Dhaka are lower than other groups of the Dhaka city and the families mean monthly household income 6833.33 BDT when the whole country's per capita mean monthly income was 8869.50 BDT (1314 US Dollars/year) (bdnews

Sources		Percentages N (%)	Morbidity Patterns			
			yes (n)	(n %)	No (n)	(n%)
For drinking water	Supply water	42 (41)	31	73	11	27
	Tube well water	28 (28)	16	57	12	43
	Cannel/river water	00 (00)	0	0	0	0
	supply & Tube well water both	32 (31)	31	96	1	4
	Total	102(100)	78	75	24	25
For household works	supply water	31 (30)	25	81	6	19
	tube well water	22 (22)	17	77	5	23
	Cannel/river water	11 (11)	10	91	1	9
	supply & Tube well water both	38 (37)	26	68	12	32
	Total	102 (100)	78	79	24	21
For Human waste disposal	inside the house	00 (00	0	0	0	0
	near outside the house	51 (50)	43	84	8	16
	far away outside the hourse	36 (35)	23	63	13	37
	near outside the house & far away outside the house both	15 (15)	12	80	3	20
	Total	102 (100)	78	75	24	43
For house hold waste dispos al	Inside the	02 (02)	2	100	0	0
	house Near outside the house	61 (59)	49	80	12	20
	Far away outside the house	26 (26)	17	65	9	35
	Near outside the house & far away outside the house both	13 (13)	10	76	3	24
	Total	102 (100)	78	80	24	21

Source: (author's own construction and calculation according to own data source).

Table 15: Relationship between morbidity patterns and using water, human waste disposal and household waste disposal status (N=102).

24.com, 2015) and these poor amount of monthly income are affecting their health, sanitation and nutritional status [14]. According to the Universal Salt Iodization Survey, 2005, millions of children are suffering from one or more types of malnutrition including underweight, Vitamin A deficiencies, and iodine deficiency disorders (IDD) and anemia. According to FAO nutritional country profile-Bangladesh (1999), the children were suffering from higher rate of micronutrient deficiencies including vitamin-A, iron, iodine and zinc deficiency in Bangladesh [21]. The present study reveals that the children intake less energy than their requirement (see Table 8). Overall their energy balance is negative, protein and iron intake is 16.55 gm and 5.70 mg daily which are too low and not fulfillment of RDA (see Table 9). Table 11 shows that the protein intake of the children are gradually decreasing day by day compared to the WHO-2000 and WFP-2008 survey with the current research but the energy intake is slightly increased. The present study shows that the energy intake ratio of the children is fluctuated but not a big margin than the other study (Table 11). Protein intake is decreased because of poverty, high price of rice and other essential commodities, low purchasing power of the children's families especially in slums children's families of Bangladesh might limit the consumption of protein. Protein intake is decreased time after time and current study shows that children intake protein 16.6 g/day but 15 years before it was 26 g/day. The insufficient consumption of protein and micronutrients results in various long and short-term health problems e.g., stunting, underweight, wasting, osteoporosis and low bone-mass [14,22].

The report of Child Nutrition Survey 2005, published by Bangladesh Bureau of Statistics, the prevalence of underweight was reported to 39.7% whereas the present study says that it is 31.8% undernourished in Slums of Dhaka city. Malnourished children are at high risk of morbidity and mortality [23]. Morbidity such as diarrheal disease, upper respiratory infections, and blindness, skin disease can significantly reduce if children use safe water, proper sanitation and hygienic disposal of human waste (Health, Population and Nutrition Sector Development Program, 2011-16). The present study shows that 76.4% of the children are suffering from diseases of last one month including 20%, 31% and 12% of the children are suffering from diarrhea, fever and skin diseases respectively (see Figures 2 and 3) and these are the common issues for children in slums of Bangladesh. The present study indicates that there are a big relationship between the morbidity patterns (last one month) of the children related with BMI and 91% of undernourished children are suffering from any sort of diseases whereas 72% of the BMI normal children are suffering from any sort of diseases last one month (see Table 12). The research shows that the underweight children's morbidity rate higher than the other BMI group children. But there is no significant difference found between BMI and the morbidity pattern of the children. According to the table 15, there are a co-relationship found between morbidity patterns and using water, human waste disposal and household waste disposal status of the children. The NHDSBD survey-2011 indicated that tube wall water and supply water are the most common sources of drinking water in Bangladesh and 94.4% of household use supply or tube well water as drinking water [24]. The present study illustrates that 41% children's family are using supply water for drinking and 28% of the children's family are using tube wall water and 31% family are using supply & tube wall water both for drinking. On the other hand, 11% of the children's family is using cannel/river's water which is so much dirty and it is not permitted for using. The current study shows that almost two-third of the children are using non-sanitary latrine which is 59% whereas 33% are using sanitary latrine. But 8% children are using sewer (open drain) which is very harmful for environment as well as children's health (see Figure 4). Table 15 indicates that the relationship between morbidity patterns and using water, human waste disposal and household waste disposal status of the children. According to Table 15, 75% children suffered from disease with related to types of drinking water. On the other hand, 79% of the children suffered from disease with related to household used water. Similarly, 75% and 80% children's disease also related with human waste disposal and household waste disposal respectively.

Overall, in this study find out that one-third of the total children are suffering from malnutrition because of their lack of nutrients intake, BMI is poor than the standard references and their pitiable socio-economical, demographical condition as well as poor sanitation and lack of hygiene practice affecting nutritional status of the slum children in Dhaka, Bangladesh.

Recommendations

The nutritional status of the slum children are more than average in Bangladesh but they lack in nutrient intake food along with physical activities and morbidity rate, this action may occur these children from their future unexpected problem during motherhood and hamper the cognitive development. Nutrition education programs need to be implemented to improve the nutritional status. The present study has generated information which indicates that the nutritional status of this group should not be overlooked and suitable approaches designed to improve their nutrition should be considered. It should studies elaborately in future. Depending on the findings, however a set of recommendation has been made for the improvement of their nutritional status.

Primary focus on nutrition promotion, and the central role of the school

Health promoting schools may provide an appropriate framework for enhancing nutrition among children, at least for those who are in school. School-based programs may also encourage children to remain in school, e.g., school-feeding programs. This is particularly important for children. Community-based institutions e.g., youth groups, community clinic, local NGOs can also be involved, in addition to using media [25].

Prevention and management of nutritional problems and risks

Health care providers can deal most directly, and primarily with micronutrient deficiencies, malnutrition of the children's.

Nutritional assessment

Nutritional assessment should be an inherent part of preventive health care services to children. This includes anthropometry could even be regularly measured in schools or if possible in house by the Government body or NGOs [26].

Provide hygiene and health related knowledge

Hygiene and health related proper knowledge should be provided and developed by the Government body or national and international NGOs. Because the study children's morbidity rate is very high and hygienic practice is too poor. One of the important things is that their using drinking and household water should be changed [27].

Control of diarrheal diseases

Issues regarding implementation of micronutrient malnutrition as

well as diarrheal diseases control programs are only partially addressed. Higher allocation of resources needs to be made to improve the outreach of health care delivery.

Control of micronutrient deficiencies

Iron deficiency anemia need to be controlled and prevented in children. Iron deficiency is the predominant cause of anemia, and correcting it is an investment in adult productive and reproductive lives. Multiple mineral vitamin supplementations to correct the problem could be done for little cost. Providing free or price-subsidized micronutrient fortified food during school hours would be a further step towards improving their nutritional status. Young children's are ideal targets for food-based approaches to improving micronutrient status, in particular vitamin A and iron [29].

Improve socio-economic and environmental condition

High socioeconomic and good environmental conditions are the most important factors associated with lower prevalence of malnutrition. The Government and the associated stockholders should be taken the proper steps for improving their socio-economic and environmental status.

To start community nutrition program

Community nutrition is a branch of human nutrition focuses on serving all people, especially in children and to meet their food and nutrition. Also needs to improve or maintain in a healthy state, through all stages of life of the children.

Perceptions and knowledge

A concentrated approach to improve awareness among all regarding the importance of nutrition for children is likely to be very useful and breastfeeding practices should be encourage among the lactating mothers. Gender discriminations should be eliminated from the society through awareness rising to ensure intra-household food security.

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Perception of Athletes about Diet and Its Role in Maintenance of Sports Performance

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Abstract

The focus of this research study was to assess the perception of athletes about diet and its role in the maintenance of performance. The target population of this research study was comprised of all the players of different sports clubs of District Bannu. Two hundred and six (206) players were randomly selected as sample of the study. For the collection of data, the researcher developed a closed form of questionnaire. The developed questionnaire personally served by the researcher among the respondents and collected back after getting it filled by the respondents. The collected data were tabulated and analyzed by using percentage and mean average as statistical tool. After data analysis, the researcher arrived at conclusion that proper diet is the basic requirement of athletic performance. The data also revealed that carbohydrates, protein, fats, vitamins, minerals and water are more important to consume in proper amount for sports participation.

Keywords: Athlete; Diet; Training; Sports performance

Introduction

Sports diet has been one of the basic needs of every sportsmen participating at various level of sports. Sports diet has the variety of benefits for maintenance and improvement of performance for an athlete. The performance of sportsmen is decreasing day by day. There may be many reasons behind this decreasing standard. Diet is one of the basic requirements for sportsmen. How much diet is necessary for athlete and is sufficient diet is provided to athlete before, during and after the competition? For the purpose to discover the fact that the researcher, intend to conduct a research study under the title "Diet and Sports Performance".

Diet and Sports Performance

According to Hoch et al. [1], the term diet refers to the collection of such type of food which helps to improve the physical condition, controls weight and helps to cure diseases by making the immune system strong. Similarly, Khan [2] defines the sports diet as the diet, which need by athlete before, during and after the activity.

Every athlete need to use proper diet before, during and after the activity. Lacking of proper diet not only adversely affects the performance of athlete but it significantly affects the overall functional capacity of the body of athlete [2].

Sports diet has been one of the basic needs of every sportsmen participating at various level of sports. According to Hoch et al. [1], sports diet enhances athletic performance by decreasing fatigue and the risk of disease and injury; it also enables athletes to optimize training and recover faster. Athletes must fuel their bodies with the appropriate nutritional foods to meet their individual energy requirements in competition, training and recovery. If these nutritional needs are not met, there is an increased risk of poor performance and health issues.

Sports diet is a strong and valuable tool for promoting the athletic performance. It is an energy source for our body, which gives us to "get up and go." Without sports diet an athlete, remain unable to show and to maintain performance during the competition. For the maintenance of performance a player need to used different nutrients such as carbohydrates, protein, and fats etc. [3].

Sports diet must be consist of food nutrients, which are more beneficial such as according to Litte. Carbohydrates should be the largest percentage of an athlete calorie intake, at least 50% to 60% in his food. This helps to meet with the demands of energy needed during exercise, maintain blood glucose and refill muscle glycogen stores.

According to Lemon [4], protein is required for the hormone and enzyme production, nutrient transfer in the blood, connective tissue support, and the repair of tissue in response to periods of exercise. They should consume 10-15% of total calories from protein. Similarly Cotugna et al. [5] stated that Fats intake is important for the energy production, protecting organs, providing insulation to the body, and facilitating fat-soluble vitamin uptake and essential fatty acid intake.

Food components may be classified as macronutrients and micronutrients. It is necessary for athlete to use both macronutrients and micronutrients. Lacking of both macronutrients and micronutrients may cause the poor or week performance of athlete during the competition [2]. Macronutrients are essential for players in energy production, bone health, immune function and antioxidant activity. Micronutrient itself does not provide energy but helps to maintain the functional capacity of the body of athlete [6].

Diet refers to the collection of such type of food, which helps to improve the physical condition, controls weight and helps to cure diseases by making the immune system strong [1]. Use of diet directly linked with physical structure and intensity of the activities which is to be performed because some activities are high powered in nature such as football, tennis, and hockey, requiring large amounts of work, rapid movements, and more energy while others are more endurance-based, such as cross-country running and triathlon training, which require

continual lower force outputs for longer durations. Diet may be given to athlete according to the nature and type of activity. Many athletes loss performance due to improper use of diet [7].

Research Study conducted by Frank et al. [7] shown that athlete needs to use diet according to the physiological demands of his or her sport. Such as

1. Muscles which produce high tension need more protein for recovery.
2. Muscles which involve in the activity of high volumes and intensity need more carbohydrates to refill glycogen (sugar) stores.
3. Additionally, active bodies that produce large amounts of heat need more water to regulate the body temperature during the practice or competitive event.

According to Boyle [8] and Swinburn and Ravussin [9], it is necessary for a coach to identify the actual demands of sports along with the added challenges of practice and training. It is important for athlete to understand the differences in required diet for health, fitness, and athletic performance. The author further stated that food, which selected should serve to prevent nutrient deficiencies or excesses that may decrease the risk of developing health-related problems or diseases. In moderate physical activity, a healthy diet should prevent weight gain, help to maintain appropriate body composition, and prevent any adverse health issues.

The primary need for the diet of the athlete is to meet the additional nutrient requirement imposed by the training load. Different kinds of physical activities demand different levels of energy. To fulfill the nutritional need of body before, during, and after the activity or training program it is needed to know the recommended intake of nutrition [10,11].

Guidelines for taking food nutrients for performing different physical activities

According to Khan [2], it is necessary for athlete to give proper concentration to the following points as guidelines for taking food nutrients for performing different physical activities:

- Proper or adequate amount of diet helps in maintaining of health and performance. Therefore, it should be taken according to the need of the body.
- High amount of Carbohydrates should be taken for maintaining of energy level before, during and after the activity.
- After the activity, proper concentration should be given to the right intake of major nutrients for maintenance of performance.

Recommended nutrients for short, moderate and long duration sports events

It is shown by studies conducted by Boyle [8], Swinburn and Ravussin [9] and Khan [2] shown that dietary need of athlete vary from activity to activity. The following few tables shown the dietary need of athlete during, before and after the activity (Charts 1-3).

Chart 1: Recommended nutrients for short duration events players.

Nutrients	Percentage (%)
Carbohydrates	60
Proteins	15-25
Fats	15-25

Chart 2: Recommended nutrients for moderate duration events players.

Nutrients	Percentage (%)
Carbohydrates	55-60
Proteins	15-25
Fats	15-20

Chart 3: Recommended nutrients for long duration events players.

Nutrients	Percentage (%)
Carbohydrates	60-70
Proteins	10-15
Fats	20-30

Pre-Training Diet, During Training Diet and Diet after Training

Sports diet is provided in three main areas:

- Pre-training diet;
- During training diet;
- Diet after training.

Pre-training diet

The diet which is required for an athlete before participating in any kind of training program is known as pre-training diet. Pre-event diet provides energy and strength needed to finish strong. Pre-event or training diet should be high in carbohydrate, adequate in protein, and moderate in fat and fiber. A larger food should be consumed 3-4 h prior to exercise to build and maintain energy stores, while a small snack 30-60 min before exercise will provide a last minute boost of energy to the body. Hargreaves [12] of the view that consumption of a high carbohydrates diet 3-7 days before the competition may be more appropriate which may contains:

- Carbohydrates 70-80%;
- Protein 10%;
- Fats 10-20%.

Diet during training

The diet which utilized by an athlete during sports performance is called during competition diet. Sport activities that extend over a long period may require mid-activity refueling in order to enhance performance. Easily digestible, carbohydrate rich foods can help maintain sufficient energy levels throughout the duration of exercise. According to Bonci L, that Carbohydrate is needed to provide energy during exercise. It is a fact that carbohydrates are stored mostly in the muscles and liver. And during activities the store carbohydrates are utilized for the production of energy for sports performance.

During sports performance full meal cannot be consumed. However, a small, high-carbohydrate snack will need to be consumed along with adequate fluid intake from sports drinks and water. Generally, in these situations the athlete would not want to consume more than about 300 calories. The main focus is to keep the athlete hydrated and not feeling hungry, yet still leave the gastrointestinal tract empty when competition begins.

Post-event diet or diet after training

The diets, which need by athlete after performing sports activities, are known as post competition diets. Post events or training diet

needed by the body to recover the body from fatigue and to adopt the load of activities. Refilling the body's energy and nutrient stores is needed immediately after high-intensity physical activity. Post-event diet should provide carbohydrate in order to restore energy losses from exercising and also protein to assist in energy restoration, in addition to muscle tissue repair and development. Fluid and electrolytes (sodium and potassium) lost in sweat should also be restored.

Methods and Materials

Methodology is the systematic, theoretical analysis of the methods applied to a field of study. It comprises of the theoretical analysis of the body of methods and principles associated with a branch of knowledge [13]. The researcher adopted the following procedures for the purpose to collect the required data, to analyze the collected data and to draw the conclusion.

Population of the study

According to Kothari [11], "A research population is generally a large collection of individuals or objects that is the main focus of a scientific query". The population of this study was comprised of all the players participated in different sports clubs at different level of sports activities in the locality of District Bannu Kp Pakistan.

Sample and sampling size

"Sample size is an important feature of any experiential study in which the goal is to make inferences about a population from a sample. In practice, the sample size used in a study is determined based on the expense of data collection and the need to have sufficient statistical power" [14]. There are total 72 sports clubs in District Bannu KP Pakistan and the total one thousand and twenty eight (1028) players are registered in these sports clubs. It is very difficult to contact each and every player of the population. So the researcher confined his population and taken randomly two hundred and six players by twenty percent (20%) of total population. The below chart shows the detail of sample and sample size (Chart 4).

S. No.	Game/Event	Total club	Total players
	Football	19	304
	Cricket	22	352
	Hockey	8	128
	Basketball	4	40
	Volley Ball	16	192
	Table Tennis	3	12
Total		72	1028

Data collection tool

For the collection of the required data, the researcher developed structured and pre-tested Diet and Sports Performance Scale (DSPS) of three-options i.e., agree, Undecided and Disagree. The code and weight of each option.

S. No.	Option	Code	Weight
1	Agree	A	1
2	Undecided	UN	2
3	Disagree	DA	3

Before using of the developed Scale for the collection of data, the develop Scale was made reliable and valid such as:

Validity of the instrument: A 20 items likert type scale was developed to evaluate the perception of athletes about diet and its role in sports performance. In developing the instrument and to make it valid numerous methods were used. For construct validity (construct validity evaluates the degree to which the scores from the scale link to other methods of theoretical characters [2] and content validity (includes assessments of the degree to which the content of a scale relates to what it is intended to measure [2]. A variety of previous scales Burke [10], Hoch et al. [1], Boyle [8], Swinburn and Ravussin [9] was used.

Reliability of the instrument: Instruments are believed to be reliable when they are producing similar results each time when they are used [2]. The author further elaborates that instrument's reliability is determined with one or more generally accepted procedures. In estimating the internal consistency reliability, the scale was administered to 20 experts in the field of sports sciences, physical education, and education. The responses of the experts were gathered and calculated the inter item correlation on items, and the co-efficient alpha on the whole scale. The Cronback alpha of the scale was measured as 0.87 which is highly reliable.

Mode for data collection

The developed scale was personally distributed by the researcher among the selected population and collected back after getting it filled by the respondents.

Presentation and Analysis of Data

Data collected form the respondents were analyzed by using percentage and mean average as statistical tool. The analysis is shown in Tables 1-4.

$H_0$1: There is no significant provision of diet before competition as perceived by athletes

Table 1 shows that there is significant provision of diet before competition as perceived by players because 71.92% players are agree, 8.94% are undecided and the percentage of disagree is 19.13 (71.92>8.94 and 19.13). While null hypothesis stating that there is no significant provision of diet before competition as perceived by the players. So hypothesis no. 1 is rejected.

$H_0$2: There is no significant provision of diet during competition as perceived by athletes

Table 2 shows that there is significant provision of diet during competition as perceived by players because 79.5% are agree and undecided are 4.5% and percentage of disagree is 16 (79.5>4.5 and 16). While null hypothesis stating that there is no significant provision of diet during competition as perceived by the players. So hypothesis no. 2 is rejected.

$H_0$3: There is no significant provision of diet after competition as perceived by athletes

Table 3 shows that there is significant provision of diet after competition as perceived by players because the percentage of agree is 77.2 and undecided are 6.5% and disagree are 16.3% (77.2>6.5 and 16.3) While null hypothesis stating that there is no significant provision of diet after competition as perceived by the players. So hypothesis no. 3 is hereby rejected.

$H_0$4: There is no significant diet provided during training as perceived by the players

Table 4 shows that there is significant diet provided during training as perceived by players because the mean of agree is 76.2 and undecided

S. No.	Diet provided pre-competition	Agree	%	Undecided	%	Disagree	%
1.	Do you aware about the importance of pre-competition diet	102	78.46	08	6.15	20	15.38
2	Food consists of all required nutrients for sports are provided pre-competition. Do you agree?	82	63.07	28	21.53	20	15.38
3	Do you agree that sufficient amount of diet is provided before competition?	50	38.46	05	3.84	75	57.69
4	Carbohydrates are an important food nutrient to be taken before competition	112	86.15	13	10	05	3.84
5	Proper carbohydrates are provided for athlete before competition	110	84.61	12	9.23	08	6.15
6	Protein is essential for heavy training session	90	69.23	15	11.53	25	19.23
7	Protein is provided before sports competition	107	82.30	07	5.38	16	12.30
8	Fatty acid is provided in food for the maintenance of endurance performance	95	73.07	05	3.84	30	23.07
Percentage (Total)		**748**	**71.92**	**93**	**8.94**	**199**	**19.13**

Table 1: Showing the provision of diet before competition.

S. No.	Diet provided during competition	Agree	%	Undecided	%	Disagree	%
1	Easily digestible form of energy is important during sports competition	116	89.2	7	5.4	7	5.4
2	Carbohydrate is the basic food nutrient provided during sports competition	92	70.8	3	2.3	35	26.9
3	Carbohydrates like juice, sugarcane are provided during competition	122	93.8	0	0.0	8	6.2
4	Glucose is providing to an athlete during competition	85	65.4	5	3.8	40	30.8
5	Food consist of fluids with suitable nutrients is provide during participation	115	88.5	2	1.5	13	10.0
6	Proper amount of vitamins and glucose are provided during competition to an athlete	90	69.2	18	13.8	22	16.9
Percentage (Total)		**620**	**79.5**	**35**	**4.5**	**125**	**16**

Table 2: Showing the provision of diet during competition.

S. No.	Diet provided after competition	Agree	%	Undecided	%	Disagree	%
1	The use of high amount of carbohydrate is important for an athlete after competition	103	79.2	7	5.4	20	15.4
2	Carbohydrates are provided to athlete after competition	105	80.8	8	6.2	17	13.1
3	Vitamins and minerals are provided after sports competition	95	73.1	10	7.7	25	19.2
4	Refilling of athlete body's energy is the major function of post-competition diet	105	80.8	5	3.8	20	15.4
5	Protein is also an important part of post competition diet	112	86.2	3	2.3	15	11.5
6	Diet is provided in sufficient amount to athlete after competition	82	63.1	18	13.8	30	23.1
Percentage (Total)		**602**	**77.2**	**51**	**6.5**	**127**	**16.3**

Table 3: Showing the provision of diet after competition.

S. No.	Variables	Agree %	Undecided %	Disagree %
1.	Diet provided before competition	71.92%	8.94%	19.13%
2.	Diet provided during competition	79.5%	4.5%	16%
3.	Diet provided after competition	77.2%	6.5%	16.3%
Mean (Total)		**76.2**	**4.75**	**17.14**

Table 4: Showing the mean average of perception of players regarding the provision of diet before, during and after the competition.

is 4.75 and mean of disagree is 17.14 (76.2>4.75 and 17.14) while null hypothesis stating that there is no significant diet provided during training as perceived by the players. So hypothesis no. 4 is hereby rejected.

Finding and Discussion

Majority of respondents opined that proper diet is given to players before the activity. The study conducted by Clark et al. [14] supported the findings of the present study because they concluded that sufficient intake of diet before the activity is necessary for the maintenance of performance of athlete. The findings of the study conducted by Maughan et al. [15] also inline of the present study. According to the findings of that study good performance need good diet to use before the competition.

It is find out by the present research study that proper diet is given to player during the activity. Finding of the study conducted by Casey and Greenhaff [16] illustrated that preservation of athletic performance is totally depend upon the diet given during the activity. The study conducted Iglesias-Gutiérrez et al. [17], shows that such type of diet is given to athlete during the competition, which provide easily digestible form of energy.

It is perceived by the researcher that that proper diet is given to players after the activity. This finding is inline of the studies conducted by Dunford [18], Storlie [19]. According to their research study it is

necessary to provide adequate relaxation and diet to player after activity which helps them to recover the lost energy.

Conclusion

On the basis of finding, the researcher concluded that significant diet is provided to athletes during training. The data revealed that carbohydrates, protein, fats, vitamins, minerals and water are more important to consume in proper amount for sports participation.

In addition, the researcher found that food consists of sufficient amount of energy with carbohydrates, fats, protein and other micronutrients are provided before, during and after competition to athletes.

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Production of a Functional Tea from *Moringa oleifera* LAM Leaf Powder: Optimization of Phenolic Extraction Using Response Surface Methodology

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Abstract

Overproduction of free radicals is implicated in the pathogenesis of most chronic diseases. Antioxidant phytochemicals thus play an important role in the prevention of these diseases. Tea is consumed across different cultures and is a major source of phenolic compounds in our diets. The aim of this study was to optimize extraction conditions for the preparation of a phytochemical-rich *Moringa oleifera* functional tea with antioxidant potential using Response Surface Methodology. Extraction conditions (temperature (°C), solid to liquid ratio (mg/mL) and time (min)) were optimized for recovery of total polyphenols (TP), total flavonoids (TF) and total tannins (TT). At the optimal conditions, *Moringa* tea was produced and its antioxidant capacity determined using the 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging and total reducing power assays. The amount of phytochemicals extracted was significantly ($p<0.05$) influenced by the extraction variables and their interactions in some cases. All 3 response variables (TP, TF and TT) exhibited the same optimal extraction conditions which were a solid to liquid ratio of 1/20 mg/mL, a temperature of 97°C and time of 35 min with optimal yields of 56.96, 34.66 and 3.53 mg/100 mL for TP, TF and TT respectively. *Moringa olifera* functional tea prepared at these optimal conditions was able to inhibit 81% of free radical using the DPPH assay, slightly higher than ascorbic acid (77%) used as reference; and had a reducing potential of 1.75 g Ascorbic Acid Equivalence/100 g DM. The tea could thus be considered an antioxidant rich tea for use in the prevention of chronic diseases.

Keywords: *Moringa oleifera* functional tea; Total polyphenols; Total flavonoids; Total tannins; Antioxidant potential; Response surface methodology

Introduction

The rising incidence of degenerative diseases coupled with the fact that consumers have become more aware of the relationship between food and health has increased the demand for functional foods. Overproduction of free radicals or oxidant species is implicated in the pathogenesis of most chronic diseases. Excessive production of free radicals in the body can lead to oxidative damage of large biomolecules such as DNA, lipids and proteins, responsible for the pathogenesis of several chronic diseases such as cardiovascular diseases, cancers, and diabetes [1,2]. Thus, antioxidant phytochemicals can play an important role in the prevention and treatment of chronic diseases [1].

Plants with their rich bioactive compounds content (polyphenols, flavonoids, tannins, carotenoids) with antioxidant and anti-inflammatory properties beneficial in the fight against these diseases have received growing attention over the years for the production of functional foods to help fight these diseases [1-3].

Moringa oleifera Lam is a plant belonging to the Moringaceae family. Its leaves have been shown to possess antioxidant [4,5], cholesterol and lipid lowering [6], anti-tumor and anti-inflammatory [7] and anti-diabetic [8,9] properties. These properties are the result of the richness of this plant in bioactive compounds such as polyphenols (flavonoids, saponins, and tannins), carotenoids and Vitamin C [7].

Tea is a soft drink obtained by boiling and by maceration or infusion of plant materials (fresh or dried flowers, leaves, stems, roots) in hot or cold water, and is a major source of phenolic compounds in our diets given its wide consumption [10,11]. The amount of polyphenols extracted into the tea during brewing, is a function of such factors as, the time and temperature of extraction, as well as the quantity of the substrate used. *M. oleifera*, being rich in phenolic compounds, with its given health benefits could be used for the preparation of a phytochemical-rich functional tea with antioxidant potential. Such a tea could serve as a source of bioactive antioxidant phytochemicals important in the fight against chronic diseases [1,2].

Extracts of *M. oleifera* have been prepared using aqueous and organic solvents and their antioxidant effects studied [4,5,12]. However, there is the need to understand the interactive effects of the different extraction variables on phenolic contents and antioxidant properties of such extracts, and to optimize extraction conditions. In addition, when organic solvents are used these extracts cannot be consumed directly given the toxicity of these solvents. The goal of this study therefore, was, to optimize conditions for the production of a phytochemical-rich *Moringa* functional tea with antioxidant potentials using response surface methodology.

Material and Methods

Plant materials

M. oleifera leaves were harvested in Maroua in the Far North Region of Cameroon and transported to the Food Biophysics, Biochemistry and Nutrition Laboratory, of the National School of Agro-Industrial Sciences (ENSAI) of the University of Ngaoundere.

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Production of *Moringa oleifera* powder

Leaflets were detached from the *M. oleifera* leaves, sorted to remove dead leaves, washed with distilled water, rinsed and drained on plastic trays for 30 min before drying at 45 ± 2°C for 14 h in a ventilated electric dryer (Riviera & Bar QD105A, Paris, France). Dried leaves were ground in a hammer mill (Culatti, Polymix, France) and sieved through a 500 µm sieve to obtain powder. The powder samples were stored in airtight glass jars and stored at 4°C until further analyses.

Extraction of plant materials

M. oleifera powder was extracted in distilled water, using a temperature controlled shaking water bath. Solid to liquid Ratio (SLR), extraction time and temperature were varied according to experimental conditions. The extracts were filtered through whatman N°1 filter paper and the clear extracts were stored in airtight amber bottles at 4°C. Extraction was done in duplicates and the duplicate extracts pooled together. All measurements were assayed in triplicates. The extracts were analyzed for total polyphenols, total flavonoids and total tannins. Preliminary trials using one factor at a time approach was employed to determine the range of the design variables to be employed in the optimization experiments.

Experimental design

Response Surface Methodology (RSM): Response Surface Methodology (RSM) was used to optimize extraction conditions for total polyphenols (TP), total flavonoids (TF) and total tannins (TT) from *M. oleifera* leaf powder for the preparation of a functional tea. A central composite design was used to investigate the effects of three independent variables; solid to Liquid ratio (SLR or Ratio, mg/mL, X_1), temperature (°C, X_2) and time (min, X_3). The independent variables were each coded at five levels (-α, -1, 0, +1, +α) and their values were selected based on preliminary experiments. The independent variables and their coded and uncoded (actual) levels are given in Table 1. The complete design as generated by Statgraphics Centurion 15.1 software (StatPoint Technologies, Inc., Warrenton, USA) consisted of 17 experimental runs with three replications at the center point (Table 2). The response variables are total polyphenols (Y_1), total flavonoids (Y_2) and total tannins (Y_3). Experimental data were fitted to a second order quadratic polynomial model which was explained by the following quadratic equation (Equation 1):

$$\boldsymbol{Y} = b_0 + \sum_{i=1}^{k} biXi + \sum_{i=1}^{k} biiXi^2 + \sum_{i=1}^{k-1} . \sum_{j=2}^{k} bijXiXj \qquad (1)$$

$i<j$

Where Y represents the response variable; X_1, X_2, X_3, ..., X_k are the coded independent variables affecting the response Y; b_0, bi ($i=1, 2, ... k$), bii ($i=1,2 ... k$), bij ($i=1, 2, ... k; j=1,2, ..., k$) are respectively the regression coefficients for the intercept, linear, quadratic and interaction terms, k is the number of variables. The intercept, linear, quadratic and interaction terms were statistically analyzed for variation using analysis of variance (ANOVA).

Validation of model

Verification tests for the validity of the model were done using optimal extraction conditions (SLR, temperature and time) that yielded maximum values for each of the responses (total polyphenols, total flavonoids and total tannins). These conditions were obtained by solving second order polynomial models of RSM using Stat graphics Centurion 15.1 software (StatPoint Technologies, Inc., Warrenton, USA). Verification experiments were then conducted under these conditions (duplicate extractions and triplicate analyses) and the experimental and predicted values compared. The R^2 values, lack of fit test and absolute average deviation (AAD) were used to determine the validity of the model. R^2 and the test of fitness were derived from the analysis of the experimental matrix through Statgraphics, and AAD was calculated using the following formula [13]:

$$AAD = \frac{\sum_{i=1}^{p} \frac{|Yobs - Ycal|}{Ycal}}{p}$$

Where Y_{obs} and Y_{cal} respectively represent experimental and calculated responses and p is the number of experimental test.The model is valid if the lack of fit is insignificant, AAD is close to zero and R^2 is close to 1 [13].

At the determined optimal conditions, a functional tea was prepared and its antioxidant potential determined using the DPPH (2,2' diphenyl-1-picryl hydrazyl) radical scavenging capacity and Total reducing Power (TRP) assay.

Analysis of the response variables

Total polyphenols: The total polyphenols were determined by the method of Makkar et al. [14]. Extract (10 µl) was diluted 20 times with distilled water (2.99 mL) in a test tube and mixed with 500 µL of Folin-Ciocalteu reagent and 400 µL of 7.5% sodium carbonate (w/v). The mixture was votexed, and incubated in the dark at room temperature for 10 min. The absorbance was measured at 760 nm using a spectrophotometer (Metertech SP8001, Germany). Total phenolic content was calculated against a calibration curve established using gallic acid and expressed as mg gallic acid equivalent (GAE) per 100 mL.

		Coded Levels				
Independent variables	**Symbol**	**(-α) -1.682**	**-1**	**0**	**1**	**(+α) 1.682**
		Actual Levels				
Solid to liquid ratio-SLR (mg/mL)	X_1	1/61	1/40	1/26	1/20	1/17
Temperature (°C)	X_2	50	60	75	90	100
Time (min)	X_3	3	10	20	30	37

Table 1: Levels of independent variables and their coded and actual levels established according to the central composite design.

	Coded variables			Responses		
Run	**X_1 mg/mL**	**X_2 °C**	**X_3 min**	**Total polyphenols (mg/100 mL)**	**Total flavonoids (mg/100 mL)**	**Total tannins (mg/100 mL)**
1	0	0	0	46.58	20.79	2.72
2	0	0	0	47.45	22.04	2.40
3	0	0	0	46.30	20.81	2.29
4	-1	-1	-1	40.68	12.55	1.03
5	1	-1	-1	42.78	17.33	2.68
6	-1	1	-1	41.48	16.11	0.86
7	1	1	-1	51.80	27.30	1.87
8	-1	-1	1	43.44	14.95	1.48
9	1	-1	1	50.71	21.14	2.13
10	-1	1	1	42.64	16.45	2.11
11	1	1	1	53.32	29.40	3.53
12	-1.682	0	0	34.17	11.70	1.13
13	1.682	0	0	51.85	29.07	2.94
14	0	-1.682	0	42.06	12.59	2.33
15	0	1.682	0	49.30	23.09	3.12
16	0	0	-1.682	48.03	19.68	1.74
17	0	0	1.682	50.57	22.46	2.32

Table 2: Central composite design for independent variables and measured responses.

Total flavonoids: Total flavonoids were determined by a colorimetric method as described by Adom [15]. To 0.1 mL of extract was added 2.4 mL of distilled water and 0.15 mL of sodium nitrite (5% w/v) and the mixture incubated at 25°C for 5 min. Thereafter, 0.15 mL of Aluminum chloride hexahydrate (10% w/v) was added followed by a second incubation. Finally 1 mL of 1M sodium hydroxide solution was added and the optical density was read at 510 nm against a reagent blank. A calibration curve was established using catechin solution. Flavonoid concentration was calculated from the calibration curve and expressed as catechin equivalents per 100 mL.

Total tannins: Total tannins were determined using the vanillin HCl method as described by [16]. To 1 mL of extract was added 3 mL of 4% (w/v) vanillin in methanol, followed by addition of 1.5 mL concentrated hydrochloric acid. The mixture was vortexed and incubated at 30°C for 20 min. The absorbance was read at 500 nm against a blank. Tannin content was calculated from a standard curve prepared using tannic acid solution (0.2 g/L). The results were expressed as equivalent grams of tannic acid per 100 mL.

Determination of antioxidant activity of functional tea

DPPH radical scavenging activity: Antioxidant capacity (Radical scavenging activity) of *M. oleifera* functional tea was determined using the modified Brand-Williams et al. [17] method. DPPH (2,2'-diphenyl-1-picryl hydrazyl) in ethanol is a stable radical, dark violet in color. Its color is bleached by its reaction with a hydrogen donor. For analyses, 0.1 mL of *M. oleifera* tea was added to 2 mL of 100 µM DPPH solution in ethanol. Ethanol without extract was included as control. The reaction mixture was incubated for 30 min in the dark at 25°C and the absorbance read at 517 nm. Vitamin C was used as the standard against which the antioxidant activity of the tea was compared. The free radical scavenging activity was calculated as follows:

$$\text{DPPH Radical Scavenging Activity } (\%) = \frac{(\text{Abs. control} - \text{Abs. extract}) \times 100}{\text{Abs. control}}$$

Where Abs. is the Absorbance at 517 nm

DPPH activity was expressed as % inhibition.

Total reducing power

The reducing power of *M. oleifera* functional tea was determined by the method of Yen and Chen [18] using potassium ferricyanide ($K_3Fe(CN)_6$). An aliquot of extract (100 µL) was mixed with equal amounts of 0.2 M phosphate buffer (pH 6.6) and 1% potassium ferricyanide and incubated for 20 min at 50°C followed by precipitation with 10% TCA. After centrifugation at 3,500 rpm for 15 min, the supernatant was diluted with equal volumes of distilled water and 100 µL of 0.1% ferric chloride ($FeCl_3$) to determine ferric reducing capacity of *Moringa* tea. The absorbance was read at 700 nm against a reagent blank. A higher absorbance indicates a higher reducing power because more ferric cyanide is reduced to ferrous cyanide by the tea. Ascorbic acid was used as reference standard and results expressed as ascorbic acid equivalence (g AAE/100 g Dry Matter).

Statistical analysis

All analyses were conducted in triplicates. Mean values were analyzed using Duncan multiple range test, and the software Stat graphics Centurion 15.2 was used to fit the generalized second order polynomial equation (Equation 1) to each of the dependent variables. Response surface plots were generated from the polynomial models and optimum conditions identified from them.

Results and Discussion

Model fitting

Extraction conditions for total polyphenols (TP), total flavonoids (TF), and total tannins (TT) from *Moringa oleifera* leaf powders were optimized using response surface methodology and a central composite design for the production of a functional tea. Table 2 shows the experimental design and the corresponding response data for TP, TF, and TT. The experimental data was fitted to a second order polynomial equation (Equation 1), and regression coefficients for the intercept, linear, quadratic and interaction terms of the model were analyzed statistically for variation using ANOVA. The results of these analyses are presented in Table 3. Using the determined regression coefficients, second order regression equations for the concentration of TP (Y_1), TF (Y_2) and TT (Y_3) were established (Equations 2, 3, and 4 respectively), where X_1, X_2 and X_3 are the coded values for the independent variables SLR, temperature and time respectively. From Table 3 we observe that the second order regression models were significant ($p<0.05$), thus demonstrating the significance of the experimental variables. The models p values were respectively 0.0001, 0.0001 and 0.006 for TP, TF and TT. To verify the models accuracy we used the R^2, lack of fit, and the AAD. The determination coefficient R^2 which is the ratio of observed variation to the total variation is a good measure of the models overall performance. In this study R^2 for TP, TF and TT were respectively 0.96, 0.99 and 0.91. This coupled with the fact that the lack of fit was not significant ($p>0.05$) and that AAD was small (0.02, 0.02 and 0.1 for TP, TF and TT respectively) confirms the validity of the model (Table 3). The R^2 values for TP, TF and TT were greater than 90% indicating that a high proportion of the variability in the response variables can be attributed to the independent variables and that only a small portion of the variability in the response variables is due to other uncontrollable factors. Thus the model can be used to predict response values. The adjusted R^2 values were above 80%. The models were therefore judged to accurately represent data in the experimental region and were used to navigate the design space.

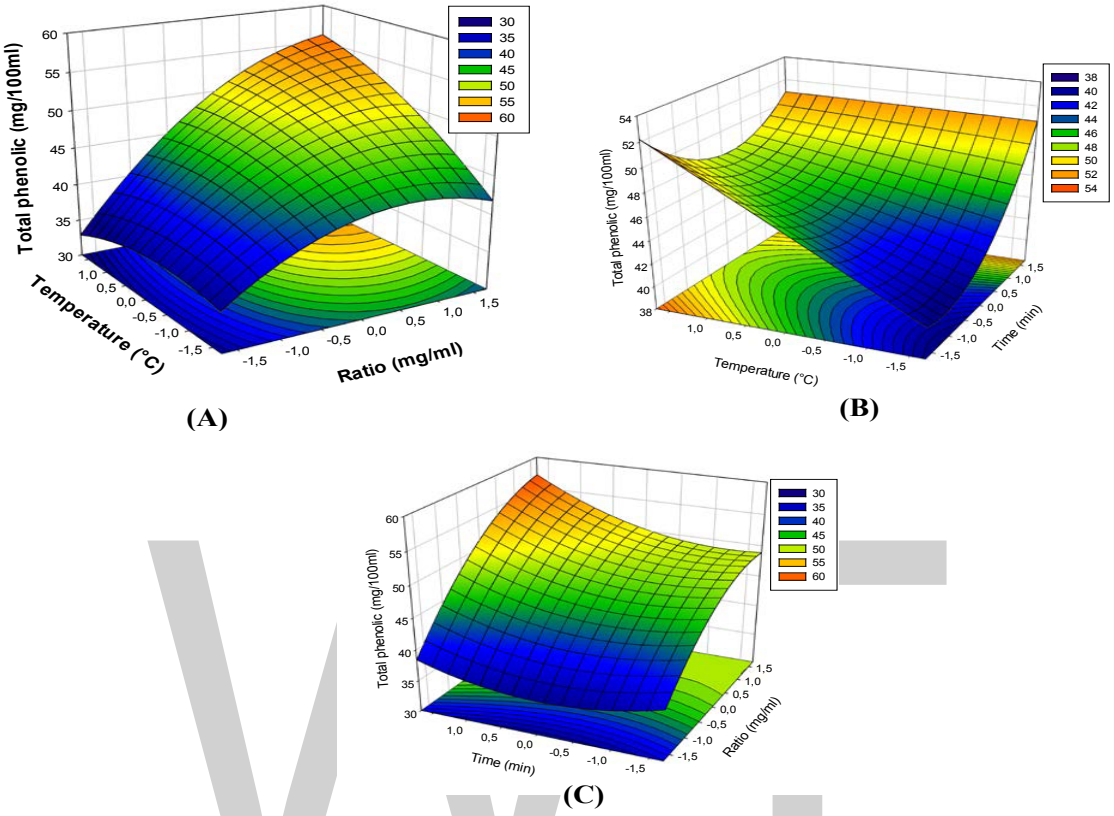
$$\mathbf{Y_1} = 46.78 + 4.40\mathbf{X_1} + 1.74\mathbf{X_2} + 1.29\mathbf{X_3} - 135\mathbf{X_1}^2 + 1.45\mathbf{X_1X_2} + 0.69\mathbf{X_1X_3} - 0.41\mathbf{X_2}^2 - 1.001\mathbf{X_2X_3} + 0.87\mathbf{X_3}^2 \quad (2)$$

$$\mathbf{Y_2} = 21.23 + 4.71\mathbf{X_1} + 2.99\mathbf{X_2} + 0.98\mathbf{X_3} - 0.35\mathbf{X_1}^2 + 1.65\mathbf{X_1X_2} + 0.40\mathbf{X_1X_3} - 1.25\mathbf{X_2}^2 - 0.47\mathbf{X_2X_3} - 0.11\mathbf{X_3}^2 \quad (3)$$

$$\mathbf{Y_3} = 2.49 + 0.57\mathbf{X_1} + 0.17\mathbf{X_2} + 0.28\mathbf{X_3} - 0.22\mathbf{X_1}^2 + 0.02\mathbf{X_1X_2} - 0.08\mathbf{X_1X_3} + 0.03\mathbf{X_2}^2 + 0.38\mathbf{X_2X_3} - 0.22\mathbf{X_3}^2 \quad (4)$$

Effect of extraction conditions (concentration, temperature and time) on response variables

Total polyphenols: The relationship between the independent variables (extraction conditions) and polyphenols is illustrated by 3 dimensional response surface and contour plots generated by the model (Figure 1). In these three variable plots when two experimental variables are depicted on the dimensional plots, the third variable is kept constant at its center value. As can be seen from Table 3, the independent variables had a significant ($p<0.05$) positive influence on TP extracted; that is the amount of polyphenols extracted increased with an increase in these variables (Figure 1). Solid to liquid ratio had the most positive linear coefficient (4.40), followed by temperature (1.74) and lastly time (1.29). However, the quadratic effect of SLR had the most negative effect (-1.35) on TP indicating that higher SLR are not favorable for polyphenol extraction. The amount of polyphenols extracted increased from a ratio of 1/61 up to a ratio of 1/20 and thereafter remained constant (Figure 1A). At higher SLR saturation



*When two extraction variables are shown on 3D plots, the third variable is held constant at its center value

Figure 1: Effect of extraction variables (solid liquid ratio-ratio, temperature and time) on total polyphenols contents. (A) Higher temperatures and ratio improved the amount of polyphenols extracted. The interaction between high temperature and higher ratios were favorable for polyphenol extraction. (B) The amount of polyphenols extracted increased with temperature and with time, though higher temperatures had a slightly negative effect at longer times. (C) At higher ratios more polyphenols were extracted but required longer times.

Source	Total polyphenols (mg/100 mL)					Total Flavonoids (mg/100 mL)				Total Tannins (mg/100 mL)			
	DF	Coefficients	Sum of squares	F-value	P-value	Coefficients	Sum of squares	F-value	P-value	Coefficients	Sum of squares	F-value	P-value
X_0, Constant	-	46.78	-	-	-	21.23	-	-	-	2.49	-	-	-
Linear	-	-	-	-	-	-	-	-	-	-	-	-	-
X_1, Ratio	1	4.40	264.52	735.52	0.0014	4.71	303.11	591.29	0.0017	0.57	4.44	88.91	0.0111
X_2, Temperature	1	1.74	41.50	115.39	0.0086	2.99	122.78	239.51	0.0041	0.17	0.41	8.23	0.1030
X_3, Time	1	1.29	22.79	63.37	0.0154	0.98	13.00	25.36	0.0372	0.28	1.04	20.92	0.0446
Quadratic													
X_1^2	1	-1.35	20.62	57.33	0.0170	-0.35	1.42	2.77	0.2379	-0.22	0.54	10.72	0.0820
X_2^2	1	-0.40	1.88	5.23	0.1495	-1.25	17.70	34.52	0.0278	0.03	0.01	0.15	0.7322
X_3^2	1	0.87	8.56	23.81	0.0395	-0.11	0.14	0.27	0.6546	-0.22	0.54	10.89	0.0808
Interaction	-	-	-	-	-	-	-	-	-	-	-	-	-
X_{12}	1	1.45	16.91	47.01	0.0206	1.65	21.68	42.29	0.0228	0.02	0.00	0.04	0.8669
X_{13}	1	0.69	3.82	10.63	0.0826	0.40	1.26	2.45	0.2580	-0.08	0.05	0.90	0.4425
X_{23}	1	-1.00	8.02	22.30	0.0420	-0.47	1.78	3.47	0.2037	0.38	0.05	22.85	0.0411
Lack of fit	5	-	14.38	8.00	0.1149	-	2.62	1.02	0.5621	-	0.67	2.68	0.2941
Pure Error	2	-	0.72	-	-	-	1.03	-	-	-	0.10	-	-
Total (corr.)	16	-	414.39	-	-	-	485.81	-	-	-	8.88	-	-
R^2	-	0.9636	-	-	-	0.9925	-	-	-	0.9136	-	-	-
Adjusted-R^2 AAD	-	0.9167 0.020	-	-	-	0.9828 0.020	-	-	-	0.8025 0.103	-	-	-

DF: Degree of Freedom; AAD: Absolute Average Deviation

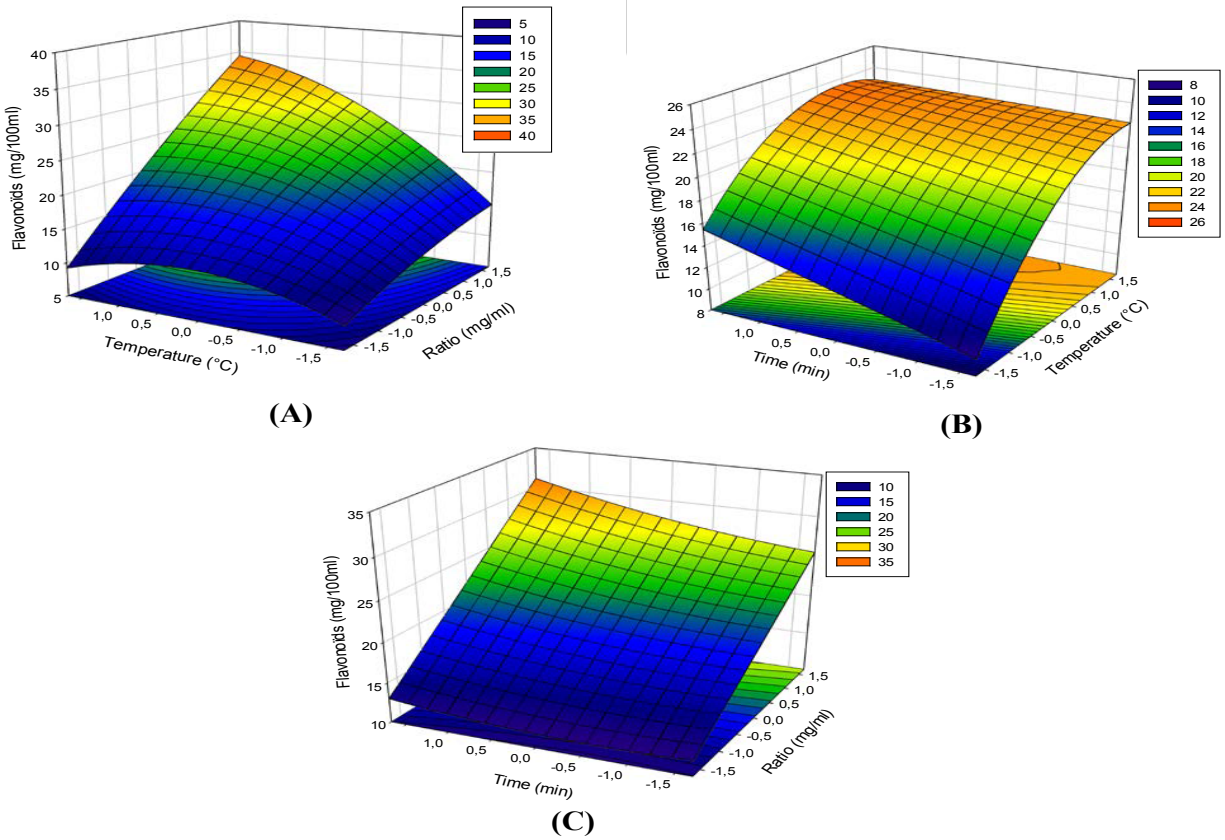
Table 3: Analyses of variance and regression coefficients of second degree polynomial models for the different response factors (Total polyphenols, total flavonoids, and total tannins).

of the medium may account for the stability in polyphenols extracted. The interaction between SLR and temperature was significant ($p<0.05$), and positively influenced polyphenol extraction, whereas that between temperature and time had a negative effect on TP. However, polyphenol extraction increased independently with temperature and time (Figures 1B and 1C). The duration of extraction is a function of the extraction temperature which influences the diffusion of extraction materials into the solvent. Increase in extraction temperature could soften the matrix and break protein-polyphenol and polysaccharide-polyphenol linkages, thus facilitating the migration of polyphenols into solution [16,19]. At higher temperatures for longer times, oxidation of polyphenols may occur [20] reducing quantities of polyphenols extracted.

Total flavonoids: All three independent variables significantly ($p=0.0001$) influenced TF extraction positively (Table 3) with concentration again having the highest linear positive coefficient (4.71), followed by temperature (3.00) and time (0.98). Thus higher SLR, temperatures and time improved flavonoid extraction (Figure 2). Dailey and Vuong [21] had observed that flavonoid extraction increased with temperature. The quadratic effect of temperature significantly ($p=0.03$) negatively (-1.25) influenced TF. With time held constant at its center value (20 min), the amounts of flavonoids extracted increased with increasing SLR and with temperature of extraction (Figure 2A) and the interaction between these two variables was significant ($p=0.02$). Total flavonoids extracted increased with temperature up to 85°C and thereafter remained constant. When SLR was fixed at its center value, TF extracted increased with temperature and time, but more so with temperature (Figures 2B and 2C). The interaction temperature time (X_2X_3) and SLR time (X_1X_3) had no significant influence on flavonoids.

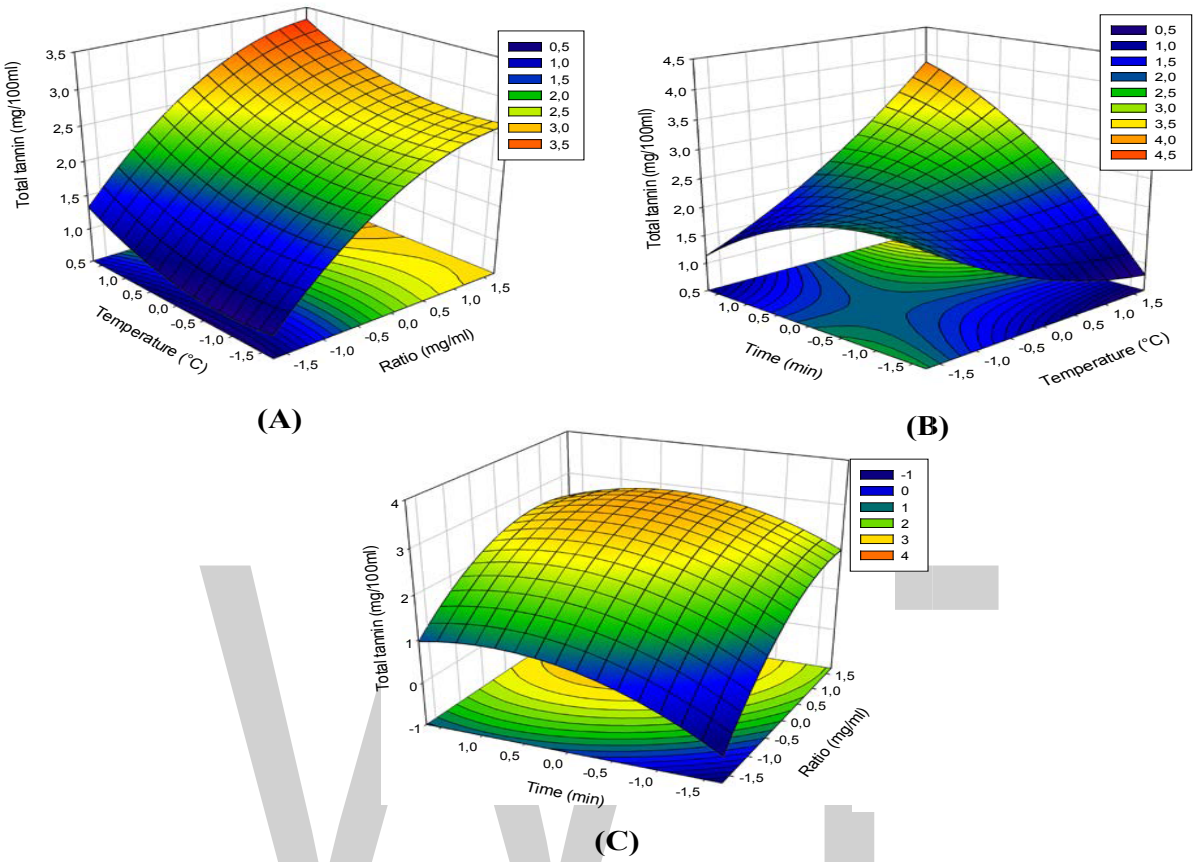
Total tannins: Only the linear effect of SLR and time significantly ($p<0.05$) influenced tannin extraction, although the effects were minimal given the small values of their coefficients which were respectively 0.57 and 0.28 (Table 3). Tannin extraction was positively (0.38) and significantly ($p<0.05$) influenced by the interaction between time and temperature (Figures 3B and 3C). At higher temperatures, longer times favored tannin extraction. Tannins extracted increased from 3 to 20 min, remained constant up to 30 min and thereafter dropped. Tannins were not appreciable influenced by the independent variables suggesting either that their concentrations in *M. oleifera* leaf powders are small or that the extraction variables do not significantly affect tannin extraction. It is evident from the above that solid to liquid Ratio and temperature had the most effect on TP and TF.

Determination of optimum extraction conditions: The optimum values for the independent variables were obtained by solving second order regression equations (Equation 2, 3, and 4) using the software Stat graphics Centurion 15.2 and the maximum desirability of all the dependent variables. It emerges from this that the optimum



*When two extraction variables are shown on 3D plots, the third variable is held constant at its center value

Figure 2: Effect of extraction variables (solid liquid ratio-ratio, temperature and time) on total flavonoids contents. (A) Flavonoids were extracted more at higher temperatures and ratios. (B) Flavonoids extracted increased with temperature and with time but peaked and remained stable at higher temperatures. (C) The amount of flavonoids increased with ratio, and with time of extraction.



*When two extraction variables are shown on 3D plots, the third variable is held constant at its center value

Figure 3: Effect of extraction variables (solid liquid ratio-ratio, temperature and time) on total tannin contents. (A) Tannins increased with ratio and temperature. (B) Time had no effect on tannins at lower temperatures but as temperatures increased, longer times favored tannin extraction. (C) Time and ratio improved tannin extraction up to a maximum and later dropped.

extraction conditions for the three measured responses TP, TF and TT are the same (1/20 mg/mL, 97°C and 35 min with desirability of 1). This observation concurs with previous findings by Khan et al. [22] in the optimization of extraction conditions of phenolic compounds from orange peel using ultrasound centered composite design. The authors noted that the optimal extraction conditions for the different polyphenols were concentrated around the same values. Similarly, the work of Ondrejovic et al. [19] on optimizing extraction of antioxidants from the leaves of *M. officinalis* showed that total polyphenols and flavonoids have the same optimal conditions, which is in accordance with our results.

To check the validity of this model, extractions were done using the optimal conditions and the response factors measured. Mean values for the response factors are presented on Table 4 alongside the predicted values. No significant differences were observed between predicted and experimental values for TP, TF and TT. We therefore conclude that the equations are adequate for predicting the response factors.

Antioxidant capacity of *Moringa oleifera* functional tea: *M. oleifera* functional tea was prepared using the optimum conditions and its antioxidant potential measured using the DPPH radical scavenging and the total reducing power assays. DPPH radical was used as a substrate to evaluate the free radical scavenging activity of *Moringa* tea. DPPH is known to abstract labile hydrogen and the ability to scavenge the DPPH radical is related to the inhibition of lipid peroxidation [23]. Reduction in DPPH absorbance implies increased antioxidant potential of the extract. *Moringa* tea thus showed strong antioxidant potential as it inhibited 81% of the DPPH radicals compared to 76.5% inhibition observed with vitamin C (0.1 mg/ml) that was used as reference (Table 4). The DPPH scavenging activity of *Moringa* tea is comparable to that (89%) reported by Pakade et al. [24] with methanolic extracts of *M. oleifera* leaves.

Reducing power is a measure of the reductive ability of a substance. It is evaluated by its ability to donate electrons and reduce Fe^{3+} to Fe^{2+}. It is thus used to measure the potential antioxidant activity of a substance [6,25]. Results showed that *Moringa* tea has good reductive capability (1.75 ± 0.21 g AAE/100 g DM). Similar observations have been reported by Shih et al. [25].

The antioxidant capacity of *Moringa* has been attributed to its polyphenols and flavonoid contents [25,26]. Several studies have also shown significant correlations between phenolic contents and antioxidant capacity [10,27].

Conclusion

This study shows that extraction conditions influence recovery of phenolic compounds and that Response surface methodology could be employed to optimize phenolic extraction from *Moringa oleifera* leaves for the production of a phytochemical-rich antioxidant functional tea. Optimum extraction conditions were the same for total polyphenols,

Independent Variables			Dependent variables (Responses)	Optimum Value	
X_1 (mg/ml)	X_2 (°C)	X_3 (min)		Predicted	Experimental*
-	-	-	Y_1 (mg/mL)	56.92	56.96 ± 0.07
1/20	97	35	Y_2 (mg/mL)	34.35	34.66 ± 0.07
-	-	-	Y_3 (mg/mL)	3.62	3.53 ± 0.03
Antioxidant Capacity					
-	-	-	DPPH (% inhibition)	-	80.94 ± 0.76%
-	-	-	Reducing power (g AAE/100 g DM)	-	1.75 ± 0.21
-	-	-	-	-	-

[a]X_1: Solid to liquid ratio; X_2: Temperature; X_3: Time; Y_1: Total polyphenols; Y_2: Total flavonoids; Y_3: Total tannins; AAE: Ascorbic acid equivalence. *Values are means of duplicate extractions and triplicate analyses (n=6); DM: Dry matter

Table 4: Optimum conditions, predicted and experimental values of response variables and antioxidant capacity of *Moringa oleifera* functional tea[a].

total flavonoids and total tannins at 1/20 mg/mL solid to liquid ratio, 97°C for temperature and time of 35 min. The interaction between solid to liquid ratio and temperature had the most effect on TP and TF, but not on TT. There were no significant differences between predicted and experimental values suggesting that the response models could reliably predict extraction conditions. *Moringa* functional tea inhibited 81% of free radicals in the DPPH assay and had reducing capacities of 1.75 g Ascorbic acid equivalence/100 g DM. Given the strong antioxidant potential of this *M. olefera* tea, its consumption could be beneficial in the prevention of stress related chronic diseases. Further investigations are however needed to determine the actual functionality of this tea.

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Freshness and Shelf Life of Air Packaged and Modified Atmosphere Packaged Fresh Tilapia Fillets during Freezing-point Storage

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Abstract

The freshness and shelf life of filleted tilapia packaged in air packaging (AP) and modified-atmosphere packing (MAP) of CO_2/N_2 30%/70% conditions, combined with freezing-point storage (-0.7°C–0°C) were investigated by sensory, textural, and physicochemical parameters, as well as microbiological and endogenous enzyme activities. The results showed that the total volatile base nitrogen, myofibril fragmentation index, sensory scores, total viable count, and pH in MAP tilapia fillets were lower than those of AP tilapia fillets, while the salt-soluble protein level and water holding capacity of MAP tilapia fillets were higher than those of AP tilapia fillets. Results showed that MAP combined with freezing-point storage exhibited a significant fresh-keeping effect on tilapia fillets, the shelf lives of AP and MAP tilapia fillets were 11th and 14th day, respectively; and total viable count and total volatile base nitrogen were 5.94 log CFU/g and 21.74 mg/100 g in MAP at 14th day, respectively. It was concluded that an application of MAP combined with freezing-point storage on tilapia fillets preservation was achieved.

Keywords: Tilapia fillets; Air Packaging (AP); Modified Atmosphere Packaging (MAP); Freezing-point storage; Freshness; Shelf life

Introduction

With increasing demand for tilapia fillets, retailers seek improved freshness and prolonged shelf life. Simultaneously, consumers demand high-quality food, with corresponding expectations of quality maintenance at a high level between the production and consumption of food. However, the shelf lives of fresh fishery products are most crucially affected by microbial activities, which are influenced mainly by the storage temperature [1]. Therefore, the food industry seeks methods to maintain the freshness and extend the shelf life of various types of fish.

The application of Modified Atmosphere Packaging (MAP) to food is an effective available method of food preservation. Use of this technology has increased recently for its effectiveness and low cost in reducing the occurrence of oxidative reactions [1]. Monteiro et al. [2] reported that the shelf life of refrigerated tilapia fillets, treated by a combination of MAP at 40% CO_2 and 60% N_2 and irradiation, was increased from 3-8 days. Erkan et al. [3] found that MAP ($O_2/CO_2/N_2$, 5%/70%/25%) storage could extend the shelf life of chub mackerel to 12 days, compared to the air-packaged (AP) and vacuum-packed shelf lives of 9 days each when stored at 4°C. Since fish is more highly perishable than meat, the product temperature is the most important factor for increasing its shelf life [4]. Freezing-point storage, in which the temperature is controlled between 0°C and the freezing point of the fish, has demonstrated efficacy for the refrigeration of fish of both marine and aquaculture origins [5]. According to Li et al. [6], the freezing point of tilapia is approximately -0.7°C. Zhu et al. [5] reported that controlled freezing-point storage at -0.7°C, combined with high CO_2 (60%) MAP, effectively maintained the quality of fresh catfish meat compared to traditional preservation methods. Freezing-point storage is better than refrigerated storage for extending the shelf life of fish; also, freezing-point storage may avoid formation of ice crystals, then keeping high water holding capacity. Therefore, freezing-point storage can maintain a better sensory, even though the shelf life is shorter than that of frozen storage. However, the lower amount of water frozen out leads to lower degrees of microstructural change, freeze denaturation, and drip loss; therefore, fish freshness and integrity can be better in freezing-point storage than that in frozen storage [4]. Based on these advantages, a storage method combining (MAP) and freezing-point storage (-0.7°C-0°C) is used in the present study to investigate the sensory, textural, and physicochemical parameters of stored tilapia, in addition to the microbial and endogenous enzyme activities. The purpose of this study is to test the efficacy of the method in maintaining the freshness and extending the shelf life of tilapia fillets.

Materials and Methods

Fish samples

Tilapias were purchased from the China Resources Vanguard Shop (Guangzhou, China) in April 2016. Tilapias were put into plastic tank (volume 50 L) and transported alive to the laboratory within 30 min. Twenty fish (735 ± 37 g) were killed by knocking head, cut the dorsal meat, skinned, and filleted. Each of the forty control fillets was packaged individually in polyethylene bags and immediately kept chilled at -0.7°C–0°C. Another forty treated fillets were placed in individual MAP bags comprising PA/TIE/PE/TIE/EVOH/TIE/PE/TIE/PE and packaged under a mixed atmosphere (MA) of 30% CO_2/70% N_2. The CO_2 and N_2 were mixed using a gas mixer (Map-D400, Senrui, Suzhou China). After packaging, the fillet packages were stored at -0.7°C–0°C and analyzed after storage for 0, 3, 7, 11, and 14 days.

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On each sampling day, six fillet packages from the control and treatment groups were removed from storage and analyzed. The physicochemical analysis for each fillet included measurements of the pH, Total Volatile Basic Nitrogen (TVB-N), Water Holding Capacity (WHC), Myofibrillar Fragmentation Index (MFI), Salt-soluble Protein (SSP) level endogenous protease activities, and other indices such as texture, sensory evaluation, and microbiological analysis.

Physicochemical analysis

pH: The pH levels of the tilapia fillets were measured using the method described by Cyprian et al. [1] with some modifications. Three grams of minced fish were placed in 27 mL distilled water and homogenized for 1 min. The pH was measured using an Accumet glass electrode attached to an Accumet Five Easy Plus pH meter (Mettler-Toledo, Columbus, USA). The measurement was performed three times per sample; the reported results are the average of the three measurements for each sample.

TVB-N: The TVB-N values were analyzed using the method of Liu et al. [7]. Five grams of minced fish were placed in 45 mL 0.6 M per chloric acid and homogenized for 2 min. The mixture was centrifuged at 10,000×g for 10 min at 4°C. The supernatant was filtered by gauze and the filtrate was subjected to Kjeldahl steam distillation with 5 mL 30% (w/v) NaOH. Five milliliters of 30% (w/v) aqueous boric acid was used as the extraction solution. After 5 min of reaction time, 0.01 M HCl was used for neutralization titration.

WHC: The WHC was analyzed following the method described by Liu et al. [8]. Five grams of minced fish were centrifuged at 10,000×g for 15 min at 4°C. The WHC was determined as the liquid loss and expressed as the weight percentage of liquid retained in the fillets, as calculated by the formula:

$$WHC\ (\%) = W_2/W_1 \quad (1)$$

Where W_1 and W_2 represent the weight of the minced fish before and after centrifugation, respectively.

SSP: The SSP was extracted from the tilapia as described by Subbaiah et al. [9], with some modifications. Five grams of minced fish were placed in 50 mL of extraction buffer A (50 mM phosphate buffer, pH 7.4) and homogenized. The suspension was centrifuged at 10,000×g for 10 min at 4°C and the supernatant was decanted. Fifty milliliters of extraction buffer A was added to the precipitate and the above steps were repeated. After the second centrifugation, the sediment was re-suspended in 50 mL extraction buffer B (100 mM phosphate buffer including 1.1 M NaCl, pH 7.4) and held at 4°C for 90 min. The suspension was centrifuged a final time at 10,000×g for 10 min at 4°C and the supernatant was retained. The SSP content was stained using Coomassie Brilliant Blue dye and analyzed at 595 nm by UV3000 spectrophotometer (Mapada, Shanghai, China).

MFI: The MFI was determined using a slight modification of the procedure described by Soltanizadeh et al. [10]. Half a gram of minced fish was homogenized in 30 mL isolating buffer of 25 mM phosphate-buffered saline (PBS), including 100 mM KCl, 1 mM ethylenediaminetetraacetic acid (EDTA), and 1 mM $MgCl_2$ with a pH of 7.0 at 4°C for 30 s. The mixture was centrifuged at 10,000×g for 15 min at 4°C and the supernatant was decanted. Ten milliliters of buffer was added to the sediment and the mixture was passed through four layers of gauze to remove connective tissue and debris. The filtered mixture was then centrifuged at 10,000×g for 15 min and the supernatant was decanted. This process was repeated twice. Finally, the sediment was re-suspended in 10 mL isolation buffer. The myofibril protein concentrations were determined by the Bradford method. Absorbance was measured at 595 nm. An aliquot of the myofibril solution was diluted in the isolation medium to a protein concentration of 0.5 ± 0.05 mg/mL. The diluted myofibril suspension was measured immediately at 540 nm using a UV spectrophotometer (Mapada, Shanghai, China). The measured absorbance was multiplied by 150 to produce the MFI.

Microbiological analysis

The total viable counts (TVC) of active microbes were analyzed following the method of Özyurt et al. [11] with some modifications. Five grams of fish muscle were mixed with 9 mL of Ringer's solution and homogenized for 1 min. Further decimal dilutions were made, and 1 mL of each dilution was pipetted onto the surface of plate-count agar plates. These were incubated for 72 h at 30°C. Microbiological counts were performed in triplicate and are expressed as log CFU/g.

Texture analysis

Texture profile analysis was performed using a Brookfield CT3 (Texture Analyzer, Brookfield, US) equipped with a 25 kg load cell and Texture Pro CT software. The fillets and the filleted whole fish were measured at three points, one each at the top, middle, and bottom of the fillet. A cylindrical probe of 4 mm in diameter was used to analyze the texture profiles. Testing conditions involved two consecutive cycles of 3 mm compression, cross-head movement at a constant speed of 0.5 $mm.sec^{-1}$, and a trigger point of 5 g. Texture variables of hardness 1, hardness 2, springiness, cohesiveness and chewiness were calculated.

Sensory evaluation

Both AP and MAP fish samples were analyzed following the methodology recommended by Liu et al. [8] with some modifications. The samples were evaluated by five trained panelists from the laboratory staff. The sensory evaluation was based on a ten-point scale, shown in Table 1. The scores for the separate characteristics of the fillets were summed to give an overall sensory score. The maximum score was 10 and the shelf-life criteria assumed that rejection would occur when the sensory score dropped below 4.

Endogenous enzyme extract and activity measurement

Cathepsins B and L activities: Minced fish (5 g) was homogenized in 20 mL of extraction buffer consisting of 50 mM sodium acetate (NaAc), 100 mM NaCl, 1 mM EDTA, and 0.2% (v/v) Triton X-100 (pH 4.0) for 1 min and then placed in a cell disruptor (Scientz Biotechnology Co., Ltd., Ningbo, China) for 2 min. The homogenate was then centrifuged at 10,000×g for 20 min at 4°C and filtered. The filtrate was used for the following measurements: Cathepsin B (CB) activity was determined at 30°C in reaction buffer A (pH 6.0), consisting of 352 mM KH_2PO_4, 48 mM Na_2HPO_4, and 4 mM Na_2EDTA. L-cysteine (8 mM) was added to the reaction buffer before use. CB + cathepsin L (CL) activity was determined in reaction buffer B (pH 5.5), consisting of 340 mM NaAc, 60 mM HAc, and 4 mM Na_2EDTA. Dithiothreitol (DTT, 8 mM) was added to the reaction buffer before use. A termination buffer (pH 4.3) containing 100 mM $ClCH_2COONa$, 30 mM NaAc, and 70 mM HAc was used. Z-Arg-Arg-7-amido-4-methylcoumarin hydrochloride and Z-Phe-Arg-7-amido-4-methylcoumarin hydrochloride were used as the substrates for CB and CB+CL, respectively. The reactions were initiated by adding 0.5 mL protein extract and reaction buffers A and B, respectively. The termination reagent was added last. A control using reaction buffer instead of enzyme buffer A was run in parallel. The incubation time was 30 min and the release level of 7-amino-4-

Scores	Sensory properties
10	Fresh odor and distinct meaty flavor, bright white color without mucus, stiff texture without concavities after finger pressure, clear muscle veins and compact structure
8	Normal odor, bright white color with slightly transparent or water mucus, firm and elastic texture with slight concavities after finger pressure, clear muscle veins and compact structure
6	Slight changes in odor, white color with slight dim, less elastic and slight concavities after finger pressure, clear muscle veins and flabby structure
4	Slight fishy odor, dark white color with moderate transparent mucus, soft and clammy, less elastic and concavities that disappear slowly after finger pressure, unclear muscle veins and flabby structure
2	Intense spoiled, off-odor, brownish red with slight opaque mucus, very soft and clammy, no clear muscle veins and very flabby structure
0	More intense spoiled, off-odor, yellow-green with more opaque and milky mucus, sunken and very slimy, no distinguishable muscle veins

Table 1: Sensory scheme of tilapia fillets.

methylcoumarin (AMC) was measured with an excitation wavelength of 340 nm and an emission wavelength of 440 nm. A standard curve was constructed from known concentrations of AMC in the stop buffer. The specific activity was expressed as U (units of activity) based on the release of 1 nmol AMC in 1 min. The values reported were the average of three measurements from each sample.

Statistical analysis

The results are expressed as means ± standard errors. Data on the physiological indicators, sensory characteristics, and texture profiles were analyzed by one-way analysis of variance (ANOVA), and the significant differences were compared by Duncan's multiple comparisons. The data were analyzed by the SPSS software package (Version 17; SPSS Inc., Chicago, USA) and considered very significant at a level of $p<0.01$ and significant at a level of $p<0.05$.

Results and Discussion

Physiochemical effects

The measured pH values are similar in the AP and MAP tilapia fillets, as shown in Table 2. The pH values decrease upon storage time and then increase. In the present study, the initial pH is 6.85, which is higher than those detected by Liu et al. [8], and Subbaiah et al. [9], Khalafalla et al. [12] for tilapia. According to the findings of Khalafalla et al. [12], differences in initial pH values may be due to the species, diet, season, and level of stress during the catch, as well as the type of muscle. However, further studies are required to explain the nature of the high initial pH levels. The pH in both AP and MAP fillets significantly decreases over the first 7 days, reaching minimal at pH 6.49 and 6.25, respectively ($p<0.05$). Similar results were reported by Cyprian et al. [1], Erkan et al. [3], and Sveinsdottir et al. [13] reported that MAP fish with CO_2 showed increasing exudation of the CO_2 dissolved on the fish body surface mucus, resulting in muscle acidification and decreases in the pH. Therefore, pH in MAP was lower than that of AP during the storage. Naturally, the initial reduction of pH in this study may be attributed to the decomposition of glycogen, ATP, and creatine phosphate and the dissolution of CO_2 in the fish muscle [14]. While the later increase in the pH value may relate to the production of volatile basic components, such as ammonia, by the decomposition of proteins and amino acids Subbaiah et al. [9]. Our results had similar trends with their theories. pH observed contributed significantly to SSP, springness and cathepsin L in this study, suggesting that changes of pH in the fish muscle were related to the endogenous enzymes, which may affect the degradation of salt protein. As a result, texture of fish muscle was changed.

TVB-N includes trimethylamine, dimethylamine, ammonia, and other nitrogen-containing compounds associated with seafood spoilage. The value of TVB-N increases as spoilage progresses [15]. The initial TVB-N value is 7.81×10 mg/100g of muscle, and is significantly increased to 22.05 mg/100 g and 21.74 mg/100g at 11^{th} and 14^{th} day of AP and MAP, respectively, during freezing-point storage (Table 3). These increases may be due to the degradation of nitrogen-containing compounds, such as proteins, to various amines. For many fish species, the TVB-N contents increase curvilinear or linearly with time [16,17]. The shelf lives of the AP and MAP fillets are less than 11^{th} and 14^{th} day, respectively, according to the upper limit TVB-N value of 20 mg/100 g muscle. This difference is probably caused by the CO_2 in the MAP, which inhibits the growth of microorganisms, thus effectively slowing both protein degradation and the increase of TVB-N [16]. Kaba and Corapci [18] reported that with the increase of CO_2 ratio, the TVB-N value of product decreased, suggesting that the CO_2 was responsible for delaying the formation of TVB-N by restricting bacterial growth. Liu et al. [8] suggested that TVB-N was correlated well with storage time ($r=0.98$), sensory acceptability ($r=-0.93$) and bacterial counts ($r>0.90$) during tray-packed storage at 0°C. We report similar results, the correlations between TVB-N and sensory evaluations (Table 4) are very significant ($p<0.01$). Both the sensory evaluation and TVB-N were unacceptable in AP at 11 days, but remained acceptable in MAP until 14 days.

The changes of WHC in the AP and MAP fillets are shown in Table 2. The WHC values of the samples during freezing-point storage under both storage methods are significantly increased with storage time ($p<0.05$). This may be attributed to the denaturation of proteins and the ability of proteins to bind to water become weak throughout storage, which may increase natural exudations with increasing storage time and thereby decrease the amount of water within the organism and its cells. Our results agree with those reported by Duun and Rustad [4], Liu et al. [8] and Cheret et al. [19]. Cheret et al. underscored the increase of natural exudation with time of storage; it might appear an opposite result. The WHC values of MAP fillets are lower than those of AP fillets, suggesting that AP would be weak in preserving water bonding capacity for the high speed of protein degradation. Other researchers previously demonstrated that early postmortem events, including pH decline, proteolysis, and even protein oxidation, are key indices in the ability of a meat sample to retain moisture [1,20]. Cyprian et al. [1] indicated that minor changes in the pH drastically affect the properties of the connective tissue, which directly affect the WHC of the fish muscle. Subbaiah et al. [9] proposed that the denaturation and aggregation of the main contractile proteins responsible for the functional properties of the organism are typically caused by ice crystal growth and increasing ionic strength. A weak water bonding capacity would significantly affect the sensory quality of a specimen.

Sensory attributes

Sensory scores show significant decreases with increasing storage time for both AP and MAP fillets during freezing-point storage

Physicochemical Index		Storage Time (days)				
		0 day	3 days	7 days	11 days	14 days
pH	AP	6.85 ± 0.05^{a}	6.64 ± 0.06^{b}	6.49 ± 0.02^{c}	6.53 ± 0.01^{bc}	6.58 ± 0.03^{bc}
	MAP	6.85 ± 0.05^{a}	6.72 ± 0.03^{b}	6.25 ± 0.03^{d}	6.30 ± 0.04^{d}	6.43 ± 0.01^{c}
WHC	AP	83.65 ± 0.47^{a}	88.05 ± 0.64^{b}	89.60 ± 0.65^{bc}	90.05 ± 0.59^{c}	93.75 ± 0.05^{d}
	MAP	83.65 ± 0.47^{a}	85.40 ± 0.65^{b}	85.55 ± 0.38^{bc}	86.80 ± 0.16^{c}	88.25 ± 0.20^{d}
SSP (mg/g)	AP	7.67 ± 0.744^{a}	6.89 ± 0.534^{ab}	5.43 ± 1.047^{bc}	4.86 ± 0.051^{c}	4.55 ± 0.487^{c}
	MAP	7.67 ± 0.74^{a}	7.47 ± 1.33^{a}	5.91 ± 0.62^{a}	5.26 ± 0.59^{a}	5.03 ± 0.49^{a}
MFI	AP	58.20 ± 2.2^{a}	99.40 ± 13.8^{b}	138.70 ± 4.5^{c}	212.80 ± 7.6^{d}	264.50 ± 13.9^{e}
	MAP	58.20 ± 2.2^{a}	75.60 ± 0.4^{a}	123.50 ± 17.5^{b}	162.10 ± 11.9^{bc}	190.20 ± 17.2^{c}
TVB-N (mg/100 g)	AP	7.81 ± 0.47^{a}	11.66 ± 0.60^{b}	12.81 ± 0.76^{b}	22.05 ± 0.36^{c}	30.56 ± 0.60^{d}
	MAP	7.81 ± 0.47^{a}	10.71 ± 0.81^{b}	12.60 ± 0.46^{c}	17.64 ± 0.51^{d}	21.74 ± 0.60^{e}
TVC (log CFU/g)	AP	4.24 ± 0.056^{a}	4.87 ± 0.035^{b}	5.31 ± 0.018^{c}	6.21 ± 0.023^{d}	7.24 ± 0.020^{e}
	MAP	4.24 ± 0.056^{a}	4.54 ± 0.036^{b}	5.19 ± 0.028^{c}	5.49 ± 0.028^{d}	5.94 ± 0.028^{e}
AP: Air Packaging; MAP: Modified Atmosphere-Packaged. Different letters in the same line indicate significant differences (p<0.05), n = 6						

Table 2: Physicochemical indices of AP and MAP tilapia fillets during freezing-point storage.

Texture Index		Storage Time (days)				
		0 day	3 days	7 days	11 days	14 days
Hardness 1 (N)	AP	119.50 ±9.61^{a}	117.17 ± 13.78^{a}	87.25 ± 6.88^{b}	86.17 ± 7.55^{b}	64.56 ± 5.33^{b}
	MAP	119.50 ± 9.61^{a}	118.06 ± 5.32^{a}	98.17 ± 7.61^{ab}	90.88 ± 4.06^{b}	89.83 ± 8.54^{b}
Hardness 2 (N)	AP	109.76 ± 8.99^{a}	108.00.17 ± 11.73^{a}	79.00 ± 6.54^{b}	77.08 ± 7.73^{b}	59.00 ± 4.67^{b}
	MAP	109.76 ± 8.99^{a}	104.13 ± 4.23^{ab}	84.67 ± 6.90^{bc}	80.75 ± 3.45^{c}	79.56 ± 7.76^{c}
Springiness (mm)	AP	2.55 ± 0.06^{a}	2.16 ± 0.08^{b}	2.16 ± 0.06^{b}	2.11 ± 0.04^{b}	2.06 ± 0.05^{b}
	MAP	2.55 ± 0.06^{a}	2.21 ± 0.05^{b}	2.11 ± 0.04^{bc}	2.05 ± 0.04^{c}	2.09 ± 0.04^{bc}
Cohesiveness (ratio)	AP	0.72 ± 0.02^{a}	0.65 ± 0.04^{ab}	0.63 ± 0.03^{ab}	0.57 ± 0.03^{b}	0.60 ± 0.03^{b}
	MAP	0.72 ± 0.02^{a}	0.61 ± 0.02^{b}	0.57 ± 0.01^{b}	0.57 ± 0.01^{b}	0.56 ± 0.01^{b}
Chewiness (N.mm^{-1})	AP	2.13 ± 0.44^{a}	2.05 ± 0.73^{a}	1.16 ± 0.31^{b}	1.01 ± 0.15^{b}	0.79 ± 0.25^{b}
	MAP	2.13 ± 0.44^{a}	1.65 ± 0.15^{b}	1.32 ± 0.19^{bc}	1.06 ± 0.06^{c}	1.05 ± 0.12^{c}
AP: Air Packaging; MAP: Modified Atmosphere-Packaged. Different letters in the same line indicate significant differences (p<0.05), n=6						

Table 3: Texture indices of AP and MAP tilapia fillets during freezing-point storage.

		pH	WHC	TVB-N	SSP	MFI	TVC	Sensory	Hardness	Springiness	CB	CL
pH	AP	-	-	-	-	-	-	-	-	-	-	-
	MAP	-	-	-	-	-	-	-	-	-	-	-
WHC	AP	-0.779	-	-	-	-	-	-	-	-	-	-
	MAP	-0.639	-	-	-	-	-	-	-	-	-	-
TVB-N	AP	-0.513	0.900*	-	-	-	-	-	-	-	-	-
	MAP	-0.649	0.981**	-	-	-	-	-	-	-	-	-
SSP	AP	0.836*	-0.924*	-0.870	-	-	-	-	-	-	-	-
	MAP	0.871*	-0.880*	-0.923*	-	-	-	-	-	-	-	-
MFI	AP	-0.651	0.931*	0.982**	-0.947**	-	-	-	-	-	-	-
	MAP	-0.776	0.946**	0.979**	-0.982**	-	-	-	-	-	-	-
TVC	AP	-0.628	0.944**	0.991**	-0.924*	0.995**	-	-	-	-	-	-
	MAP	-0.798	0.950**	0.970**	-0.979**	0.995**	-	-	-	-	-	-
Sensory	AP	0.543	-0.893*	-0.996**	0.888*	-0.988**	-0.989**	-	-	-	-	-
	MAP	0.590	-0.981**	-0.992**	0.887*	-0.958**	-0.955**	-	-	-	-	-
hardness	AP	0.693	-0.914*	-0.898*	0.954**	-0.941**	-0.940**	0.894*	-	-	-	-
	MAP	0.891*	-0.853*	-0.900*	0.998**	-0.969**	-0.966**	0.858*	-	-	-	-
Springiness	AP	0.892*	-0.915*	-0.719	0.838*	-0.786	-0.781	0.732	0.719	-	-	-
	MAP	0.862*	-0.828*	-0.766	0.820*	-0.802	-0.827*	0.725	0.816*	-	-	-
CB	AP	-0.769	0.349	0.033	-0.423	0.129	0.104	0.021	-0.348	-0.465	-	-
	MAP	-0.940**	0.853*	0.846	-0.946**	-0.911	0.924*	-0.798	-0.948**	-0.954**	-	-
CL	AP	-0.951*	0.730	0.528	-0.878*	0.677	0.632	-0.561	-0.773	0.762	0.773	-
	MAP	-0.997**	0.638	0.634	-0.850*	0.757	0.959**	-0.578	-0.869*	-0.882*	0.939**	-
AP: Air Packaging; MAP: Modified Atmosphere-Packaged. **Correlation is significant at p<0.01 levels (two-tail). *Correlation is significant at p<0.05 levels (two-tail)												

Table 4: Correlation between freshness indicators of AP and MAP tilapia fillets.

($p<0.05$), as shown in Figure 1. Initially, the fish freshness is excellent, but the freshness characteristics are diminished gradually with time. After 11th day, the AP fillets are found still acceptable but have a low score near the limit of score=4, and completely unacceptable at 14 (score <4), whereas MAP fillets remain acceptable at 14th day, suggesting that under the same storage time, fillets in MAP are more acceptable to consumers. The slower rate of decrease in the sensory score for MAP fillets may be attributed to CO_2 inhibiting microbial growth, slowing the rate of protein degradation, and maintaining good water retention ability. Sensory score was correlated well with WHC, TVB-N, SSP, MFI, and hardness, which may be related to juiciness, odor, veins, structure, and the softening of the sample, respectively.

Protein degradation

Variations in SSP levels of AP and MAP fillets are shown in Table 2. The initial SSP value is 7.67 mg/g fish meat, and is decreased with increasing storage time by both storage methods. This may be explained by the progressive denaturation of myofibrillar proteins during freezing-point storage; for the myofibril protein was the major component of SSP. Duun and Rustad [4] suggested that the denaturation of food muscle proteins reduces the amount of soluble proteins. Kaale and Eikevik [21] demonstrated that the variation of SSP levels during super-chilled storage could relate to different durations and speeds of blending for all experiments. A lower SSP was in MAP than that in AP during freezing-point storage, suggesting that the speed of degradation of myofibrillar proteins in MAP was lower than that in AP, indicating that the method of MAP combined with freezing-point storage on tilapia fillets may effectively prevent degradation of myofibrillar proteins. SSP was correlated well with sensory attribute ($r>0.88$), TVC ($r>-0.92$), hardness ($r>0.95$), springiness ($r>0.82$), and cathpsin B and cathpsin L, indicating SSP may be degraded by bacterial growth and endogenous enzymes, and affected sensory perception the fillets, as well as contributed to texture softening.

When muscle proteins are degraded by proteolytic enzymes, an increase in fragments of different lengths (from free amino acids to large peptides) is expected [22]. The measurement of myofibril fragmentation is reported as one of the most widely used methods to determine postmortem proteolysis by Soltanizadeh et al. [10], but is rarely used in fish, as indicated by Liu et al. [23]. The MFI shows similar trends, but is significantly higher in AP than in the MAP tilapia fillets, as shown in Table 2. The initial MFI is 51.63 and this value increases throughout freezing-point storage. Liu et al. [23] reported that the MFI of *Lateolabrax japonicus* in super-chilling combined with MAP was lower than that of non-super-chilled MAP fish, suggesting that super-chilling probably inhibited the activities of the relevant enzymes and slowed protein denaturation, thereby delaying the tenderization of flesh. MFI was correlated well with SSP ($r>-0.94$).

Microbiological analysis

The TVC from AP and MAP fillets during freezing-point storage are shown in Table 2. TVC significantly increases throughout the storage time in both AP and MAP fillets ($p<0.05$). The initial TVC is 4.24 log CFU/g, and it reaches 6.21 log CFU/g and 5.94 log CFU/g in AP and MAP fillets at 11th and 14th day, respectively. TVC of 6 log CFU/g is considered the maximum acceptable level for chilled fish Khalafalla et al. [12]. Bacteria grow more quickly in AP than MAP fillets throughout the storage time. Our results agree with those reported by Erkan et al. [3], Parlapania et al. [16] and Yesudhason et al. [24]. The difference in bacterial growth rate may be attributed mainly to the inhibiting behavior of CO_2 and the absence of O_2, as well as to the low storage temperature, which can inhibit the growth of microorganisms. Duun and Rustad [4] reported that super-chilled salmon fillets stored at -2°C in combination with MAP maintained good quality based on both sensory and microbial analyses, with negligible microbial growth for more than 24 days, whereas ice-chilled reference fillets maintained good quality for only 17 days. Parlapania et al. [16] reported that MAP affected the growth rate of spoilage bacteria, and the increase of CO_2 and decrease of O_2 by MAP inhibited bacterial growth and changed microbial spoilage by suppressing mostly Gram-negative species and favoring Gram-positive ones.

Texture analysis

Texture parameters, such as hardness 1, hardness 2, springiness, cohesiveness and chewiness are reported in Table 3. The values for hardness 1, hardness 2, springiness, cohesiveness, and chewiness are found to decrease ($p<0.05$) in both AP and MAP fillets during freezing-point storage. This indicates the gradual softening of muscle. The decreasing of springiness during storage may be related to the degeneration of protein, which decreases the binding force between muscles and the elasticity of the fish, which also indirectly reflects the decreasing quality of the fish. Hardness 1 and springiness are correlated well with SSP (Table 4), which may be explained by the denaturation of proteins and the damage of membrane structures responsible for texture. As a comprehensive evaluation parameter, chewiness is directly related to hardness and springiness, which both show decreasing trends. Excepting cohesiveness, the other texture parameters show smaller extents of decline in MAP than in AP, indicating that damage of myofibrillar proteins in AP is much more serious than that in MAP fillets. Subbaiah et al. [9] found that the softening of fish muscle during frozen storage was caused by the breakdown of the three-dimensional structures of proteins and their aggregation following the activity of proteases. Huff-Lonergan and Lonergan [20] suggested that the deterioration of fish flesh texture may relate to various intrinsic and extrinsic factors, such as the loss of water from the muscle and the destruction of muscle tissues. Protein denaturation has a profound effect on the texture qualities of meat, which is considered one of the most important sensory qualities attributes [9].

Cathepsins activities

The CB activities in both AP and MAP fillets, as well as the CL activity in AP, significantly increases at first before decreasing with increasing storage time ($p<0.05$) (Figure 2). The CL activity of MAP fillets significantly increases over time ($p<0.05$). These results partially agree with that by Gaarder et al. [25], who reported that CB and

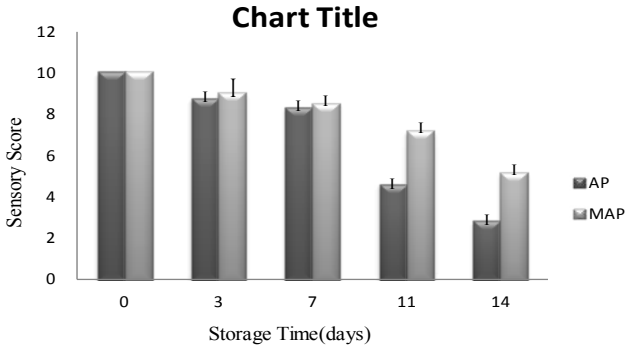

Figure 1: Variation of sensory quality in Air Packaging (AP) and Modified Atmosphere-Packaged (MAP) tilapia fillets during freezing-point storage.

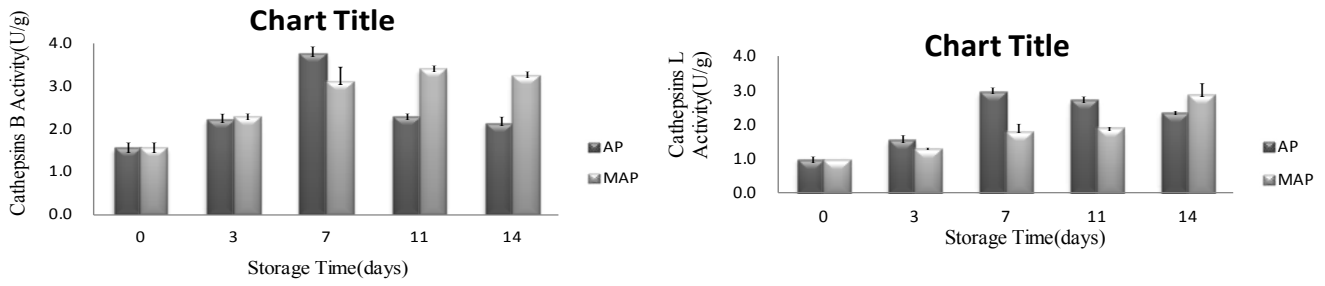

Figure 2: (a and b) depict the changes in the activities of cathepsin B and cathepsin L, respectively. One unit (U) of enzyme activity is defined as the amount of enzyme that hydrolyses the substrate to release 1 μM AMC/min at 40°C. Cathepsin activities are expressed as U/g of fish meat. cathepsin L activity was deduced by subtracting the amount of cathepsin B activity from the summed levels of cathepsin B+cathepsin L. The error bars present the standard error of the mea.

cathepsin B+L activities of Atlantic salmon (Salmo salar) fillets increased with increasing storage time both in ice-stored and super-chilled for 114 h after slaughter. The decrease in CB and CL activities from 7 days post-mortem until 14th day post-mortem of AP might indicate that the cathepsins has been active and start to autolyse and therefore the activities decreases, similar results were found in Gaarder et al. [26], that cathepsins activities decreased at 5 days post-mortem when Atlantic salmon (Salmo salar) fillets stored on ice. Cathepsins are usually located in lysosomes and thus are inactive in living tissue, because they are not in contact with their substrates. However, they may be released in the cytosol by lysosome disruption after the death of the organism [27]. Gaarder et al. [25] suggested that the principal cause of post-mortem degradation of fish muscle is the hydrolytic nature of CB and CL, and that more proteolytic peptides may be released as a result. In parallel, the texture of the fillets gradually becomes more tender, and hardness 1 and springiness are correlated well with CB and CL in MAP fillets (Table 4), suggesting that the cathepsins activities were major factors determining fillet firmness. Hardness 1 and springiness are correlated well with TVC in AP fillets (Table 4), suggesting that microorganisms may be the major factor determining protein degradation that causes the increased tenderness of tilapia fillets with increased storage time. Ayala et al. [28] proposed that partially purified resolution of rigor in the post-rigor stage increases muscle tenderization by the hydrolysis of muscle proteins by endogenous calpains and cathepsins.

Conclusion

Sensory scores, SSP contents, and texture parameters were decreased with increasing storage time. TVB-N, MFI, WHC value, and TVC increased with increasing storage time. Tilapia fillets packaged under MAP showed significant delays in deterioration. AP and MAP tilapia fillets were acceptable by sensory evaluation until the 11th and 14th days of storage, respectively. The AP and MAP tilapia fillets were also acceptable by sensory, microbiological data and TVB-N until 11th and 14th day after packaging, respectively. The extension of shelf life using the suggested gas composition could be commercially exploited. However, the gas composition did negatively impact the sensory quality of the preserved tilapia fillets.

Acknowledgments

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Household Food Insecurity and its Association with Nutritional Status among Preschool Children in Gambella Town, Western Ethiopia

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Abstract

Introduction: Food insecurity and malnutrition among children are common in developing countries including Ethiopia. Food insecurity is probably one of the determining factors of malnutrition in children but results are inconclusive.

Objectives: The aim of this study was to assess the magnitude of household food insecurity and its association with the nutritional status of children in Gambella town.

Method: A community based cross-sectional study was conducted on children in April 2016 in Gambella town, west Ethiopia. Data including household food insecurity were collected from 284 households having children 6-59 months by the face to face interview using structured questionnaire. Anthropometric measurements were measured using standard procedures to determine nutritional status of children. Descriptive statistics, bivariate and multivariate logistic regression analysis were performed to determine the association between household food insecurity and nutritional status of children.

Results: The overall prevalence of household food insecurity was 59.5% with 20.1%, 23.6%, and 15.8% households were mildly, moderately, and severely food insecure, respectively. Prevalence of stunting, underweight and wasting were 23.2%, 12.0% and 13.4% respectively. Household food insecurity was independently associated with stunting, but not with wasting and underweight after adjusting for possible confounders using multivariable logistic regression model. The odds of stunting were highly pronounced in those children who were from severely and moderately food insecure households.

Conclusion: The findings from this study suggest high prevalence of both household food insecurity and malnutrition among children in Gambella town. Household food insecurity was significantly associated with stunting. The finding implies nutrition interventions targeting children need to address household food security.

Keywords: Household food insecurity; Nutritional status; Children

Introduction

Food insecurity is a state or a condition in which people experienced limited or uncertain physical and economic access to safe, sufficient, and nutritious food to meet their dietary needs or food preferences for a productive, healthy and active life [1]. Food security, on the other hand, is achieved when all people, at all times, have physical, social and economic access to sufficient, safe and nutritious food that meets their dietary needs and food preferences for an active and healthy life [2]. Food security can be considered at national, household and individual levels. At national level, it is related to physical existence of food stocks for consumption be it from own production or from markets. Household food security is related to the ability to obtain sufficient food with sufficient quality to meet nutritional requirements of all household members. Household level food security mainly relies on household income and purchasing power of household members which again related to income distribution in the household [3].

Evidences show that the magnitude and severity of food insecurity vary across the globe with countries from developing world's having higher burden for food insecurity and its negative health consequences including malnutrition. For instance, in a study by Noreen et al. [4] undertaken to differentiate the socio-demographic risk factors between Aboriginal and non-Aboriginal households, 33% of Aboriginal households were food insecure while this was 9% for non-Aboriginal households in Canada. In Cambodia, the respective prevalences of mild, moderate and severe food insecurity were 33, 37 and 12% [5]. In Kenya the prevalence of severe food insecurity was even higher accounting for 62.5% while 11% and 8.4% were moderately and mildly food insecure, respectively [6]. Food insecurity is also common in Ethiopian households from different parts of the country. Regassa and Stoecker [7] found that the percentages of households that were mildly, moderately and severely food insecure in southern Ethiopia were 6.8%, 27.7% and 47.8%, respectively. Tamiru et al. [8] reported 59.5% of children from food insecure households were having school absenteeism in their recent study conducted on school adolescents in southwest Ethiopia. Motbainor et al. [3] also reported food insecurity to be 55.3% in households from Amhara region of Northern Ethiopia. Being one of the three underlying causes of undernutrition, household food insecurity is assumed to affect the nutritional status of children by compromising quantity and quality of dietary intake [9]. The nutritional status of preschool children is a key indicator to assess the nutritional and health status of a larger population as children are the most vulnerable to nutritional imbalances. Anthropometric indicators commonly are used to measure malnutrition in a population

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of fewer than five children [10]. The nutritional consequences of food insecurity experience include underweight, stunting and wasting, and also overweight and obesity, depending on a broad range of contextual, economic and socio cultural factors. Globally, in 2011, nearly one in four children under-five years of age (26%) were stunted while 16% were underweight. Worldwide, there has been a decline in malnutrition levels from the 1990s; however, the levels in sub Saharan Africa have remained high [11,12]. Near to 90% of stunted children in the world live in Africa and Asia, with the prevalence of stunting in Africa being 36% in 2011 [13]. According to Mini EDHS 2014, the prevalence of stunting, wasting, and underweight in Ethiopia were 40%, 8.7%, and 25.2% while these were 22.5%, 14.9%, and 18.5%, respectively in Gambella region [14]. In addition, population in Ethiopia largely dependents on an agrarian economy. However, poverty and hunger are widespread in the country despite impressive progress in agriculture in Ethiopia may be resulting in the prevalent household food insecurity [9]. On contrary to the general assumption that household food insecurity, one of the underlying causes of malnutrition in conceptual framework by UNICEF, is linked with under nutrition among children from food insecure households, some studies reported there were no associations between household food insecurity and undernutrition in children [1,5]. On top of this, no study has tried to look at the linkage between household food insecurity and nutritional status of preschool children in Gambella town despite common existence of both forms of public health problems of food insecurity and under nutrition in the same community of Gambella region. Hence, this study was aimed at assessing the association between household food insecurity and nutritionals status of children aged 6-59 months in Gambell town.

Methods and Materials

Subjects and study design

The study was conducted in Gambella Town, located at about 768 kilometers in the south west of Addis Ababa, Ethiopia. Gambella Town is the capital of the Gambella regional state. The town has been divided by five kebeles and it harbors different ethnic groups. The majority of ethnic groups residing in the town are Nuire and Agnuhak. Based on the 2007 Census conducted by the Central Statistical Agency of Ethiopia, Gambella Town has a total population of 53,022, of whom 52.6% are men. The town has a total of 11,260 households with an average of 3.8 persons to a household. The town has one hospital, one health centers, two governmental junior clinics and 15 private clinics. The livelihood of the population in the town is mainly dependent on government work and trade. The study was conducted from March 29/2016 to April 23/2016 using a community based cross-sectional study design. The study population was all children 6-59 months residing in the Gambella town.

Sample size

Sample sizes was calculated using a formula for single population proportion by considering the overall prevalence of stunting, underweight and wasting to be 22.5%, 14.5%, and 18.4%, respectively for under-five children of Gambella region that was taken from mini Ethiopian Demographic and Health Survey, 2014 [14], 5% margin of error and 95% confidence interval for each. After determining the sample size for each indicator, the largest sample size of 268 that obtained using 22.5% stunting prevalence was considered for the final sample size to have a better power in estimating population parameters. Since the overall number of study population from census was 7652 (<10,000), we used a finite population correction formula to calculate the final sample size and it became 259. Adding 10% non-response the final sample size was set to be 285.

The inclusion criteria for this study was being a child aged between 6-59-months, who dwelt in the town at least for six months, and one of the household member (either household head or caregiver) volunteer to give information. Exclusion criteria were children having severe illness and deformities which cause difficulty for anthropometric measurement during survey.

Sampling procedure

Prior to data collection, census was conducted in the all kebeles of Gambella town to get lists of household having children aged 6-59 months (census was done as part of polio campaign that was carried out by Gambella Town health office). During the census unique identification numbers were given to the households having children age 6-59 months. These identification numbers were also written on the gate of each household in order to facilitate the process of sampling technique. Then, sampling frame was prepared using these unique identification numbers given to each household having children age 6-59 months. After allocating the total sample size to each kebele of the town using population proportional to size allocation, households having children age 6-59 months were drawn using computer generated random number method using SPSS version 21.0 software. If more than one children fulfilling inclusion criteria were found in the selected household child one was selected using lottery method.

Anthropometric measurements

Anthropometric data such as body weight and length/height were collected respectively using salter scale and length board for children aged 6-24 months, and digital weighing scale that has the capacity to measure 0-140 kg (Model; SECA; 770 alpha, Hamburg, Germany) to the nearest 0.1 kg and portable height board for children aged 24-59 months. Anthropometric measurements of children were measured based on the WHO standardized procedures. Data for weight and length/height were compared to WHO standards for specific age and sex to define nutritional status of children. Accordingly, underweight, wasting, and stunting among children were defined as WAZ, WHZ, and HAZ < -2 SD and overweight as WHZ > +2 SD in the 2006 WHO growth standard [15,16]. Trained nurses did anthropometric measurements including other relevant data in the home of the selected children during day time. Height/length and weight were measured 3 times and the mean was used for analysis. Validation of instruments, and measurements and random auditing was done on a daily basis by supervisors of the study.

HFIAS measurement

Household food insecurity was measured using the Household Food Insecurity Access Scale (HFIAS) that was developed by the Food and Nutrition Technical Assistance (FANTA) project [17]. For Household Food Insecurity Access Scale (HFIAS) measurement, each of the questions was asked with a recall period of four weeks (30 days). The respondent was first asked an occurrence question-that is, whether the condition in the question happened at all in the past four weeks (yes or no). If the respondent answers "yes" to an occurrence question, a frequency-of-occurrence question was asked to determine whether the condition happened rarely (once or twice), sometimes (three to ten times) or often (more than ten times) in the past four weeks.

Household wealth index

A household wealth index was grouped by principal component analysis based on household assets and housing quality based on an earlier concept that was developed by Garenne and Hohmann to be

used as a proxy indicator for socioeconomic status of households [18]. This measurement is appropriate for urban and rural setting in low and meddle income countries. The sum of dummy variables were created from information collected on housing quality floor, walls, and roof material), availability of potable water in the compound and type of toilet facility, and ownership of household durable goods (e.g., bicycle, television, radio, motorcycle, telephone, cars, refrigerator, mattress, bed). These facilities or durable goods are often regarded as modern goods that have been shown to reflect household wealth. The scores were thus added up to give the proxy household wealth index. The index varied from 0 to 20. After checking internal consistency of each item using a Cronbach's alpha (0.79), a wealth index was rank ordered into tertiles to give low, middle and high socioeconomic status.

Data collection

Information on important child characteristics, caring practices, environmental conditions, anthropometry and socio-graphic variables were collected by face to face interview using pre-tested questionnaires adapted from related literatures. The data were collected by five data collectors (urban health extension nurses) who were supervised by two Bachelor of Science degree public health professional working in Gambella town. The supervisor provided all items necessary for data collection on each data collection day, checking filled questionnaire for completeness and consistency, and solving problems during data collection. The principal investigator did the overall supervision daily.

Data quality assurance

To insure the quality of data the questionnaires originally prepared in English was translated to Amharic and administered to respondents by local language speaking data collectors who were fluent in Amharic. The questionnaires were translated back to English to check for its conceptual equivalence. In addition, pre-test was done on 15 household having children age 6-59 months living in Itang town, which is located 38 km far from Gambella town, that wasn't included in the main study. Finally, data collection tool was refined based on the findings from the pretesting. Further, data collectors and supervisors were given a two days training to get reliable data and assure its quality. Anthropometric measurement was taken three times and the mean was taken for analysis and a difference of 100 g in weight and 0.1 cm in length was accepted as normal and also standard procedures were followed. Every morning and prior to each measurement, the weight scale was calibrated with a standard weight and instruments were calibrated according to the manufacturer's recommendations. Every day, all collected data were reviewed and checked for completeness and consistency by the supervisors. Data cleansing was done thoroughly using EpiData version 3.1 to ensure data quality for further analysis.

Data processing and analysis

Collected data were checked for completeness, consistency, and coded manually after which the data were entered into EpiData version 3.1. After this, data were exported to SPSS 21 for windows for further analysis. First univariate analysis was conducted to explore frequency distribution, central tendency, variability (dispersion) and shape of the overall distribution of different variables. Bivariate analysis was done to identify candidate variables for multivariable logistic regression model. All explanatory variables that were associated with the outcome variable at a p-value of <0.25 were considered candidates for multivariable logistic regression. Multicollinearity between different explanatory variables was checked using variable inflation factor (VIF>5) and Pearson correlation coefficient (>0.6). As food security status and food insecurity levels had collinearity with each other, they were adjusted for other explanatory variable in multivariable logistic regression model using two different models excluding one variable at a time of estimating parameter for the other variable. During each time, enter method of multivariable logistic regression model was used to identify significant predictors associated with outcome variable of interest at an Alpha level of 0.05. In multivariable analysis p-values of less than 0.05 were considered statistically significant. The results were described as Odds Ratio and 95% CI.

Anthropometric data was analyzed in WHO Anthro software. Anthropometric measurements such as height, weight, sex and age of children 6–59 months were converted into z-scores using the 2006 WHO standard growth curves. Before performing the anthropometric calculations for weight-for-height (WH), height-forage (HA), and weight-for-age (WA), the data were cleaned and the outliers were checked and they were less than 2.5 with 95% CI which is the acceptable level [16].

Ethical approval

The complete study protocol was approved by Jimma University College of Health Sciences Institutional Review Board. Permission letter to conduct the research was obtained from Gambella Regional Health Bureau and taken to each kebele admiration for their necessary support. The parent/guardian of each selected child was given brief explanations about the objectives of the study after which verbal consents were obtained from each parent guardian of a selected child. During data collection those children with acute malnutrition but didn't get appropriate care and treatment were referred to the health facilities.

Results

From the total 288 sampled households, three of them were refused to participate in the study making the response rate 98.9%. One child had missing age and height measurement. The final analysis was based on 284 study subjects. The mean (SD) ages for children from food insecure HH and food secure HH groups were 31.66 (±4.2) and 32.47 (±4.2) months, respectively. Proportions of children by their sex were almost 50% each. Nearly half of the children, 132 (46.5%) were in the age group of 36-59 months. Nuer constitute majority of the ethnic group, 78 (27.5%) followed by Agnua, 74 (26.1%). One hundred (35.2%), 55 (19.4%), 52 (18.3%) and 36 (12.7%) were Protestant, Catholic, Orthodox and Muslims, respectively. Half of mothers/caregiver attended primary education and the rest half of 143 (50.3%) had no formal education out of which, 107 (74.8%) were lived in food insecurity households and 36 (25.2%) in food secure respectively. From a total of 284 mothers/caregivers, 33 (11.6%) were not married. One hundred eighteen (41.5%) mothers/caregivers were house wife followed by governmental employee, 77 (27.1%). The mean family size was 5.7 ± 1.8. Ninety two (32.4%) households had family members with more than one under five children.

Majority, 82.7%, of households had access to high quality drinking water and latrine facility accounting for 65.8%. Almost equal proportions of households were from poor and high socioeconomic status households (Table 1).

The overall prevalence of stunting, underweight and wasting were 23.2%, 12.0% and 13.4% respectively. The prevalence of overweight was 3.5% as measured by WHZ. More than half (59.5%) of the households in our study were food insecure. Of these households, 20%, 23.6% and 15.8% were experiencing mild, moderate and severe food insecurity, respectively.

Characteristics		Food insecure N (%)	Food secure N (%)	Total N (%)
Child sex	Male	77 (55.0)	63 (45.0)	140 (49.3)
	Female	92 (63.9)	52 (36.1)	144 (50.7)
Age group of children	6-23 months	58 (59.2)	40 (40.8)	98 (34.5)
	24-35 months	33 (61.1)	21 (38.9)	54 (19.0)
	36-59 months	78 (59.1)	54 (40.9)	132 (46.5)
Age children (months)	Mean (SD)	31.66	32.47	31.99
Education (mother)	No formal education	107 (74.8)	36 (25.2)	143 (50.4)
	Formal education	62 (44.0)	79 (56.0)	141 (49.6)
Marital status (mother)	Married	137 (54.6)	114 (45.4)	251 (88.4)
	Not married	32 (97.0)	1 (3.0)	33 (11.6)
Occupation (mother)	Housewife only	84 (71.2)	34 (28.8)	118 (41.5)
	Employed	85 (51.2)	81 (48.8)	166 (58.5)
Education (father)	No formal education	55 (58.5)	39 (41.5)	94 (33.1)
	Formal education	114 (60.0)	76 (40.0)	190 (66.9)
Religion	Protestant	58 (58.0)	42 (42.0)	100 (35.2)
	Orthodox	31 (59.6)	21 (40.4)	52 (18.3)
	Catholic	40 (72.7)	15 (27.3)	55 (19.4)
	Muslim	9 (25.0)	27 (75.0)	36 (12.7)
	Other	31 (75.6)	10 (24.4)	41 (14.4)
Ethnicity	Nuer	56 (71.8)	22 (28.2)	78 (27.5)
	Agnewa	54 (73.0)	20 (27.0)	74 (26.1)
	Tigre	12 (41.4)	17 (58.6)	29 (10.2)
	Oromo	14 (40.0)	21 (60.0)	35 (12.3)
	Amhara	11 (40.7)	16 (59.3)	27 (9.5)
	Others	22 (53.7)	19 (46.3)	41 (14.4)
Family size (mean ± SD)	-	5.76	5.69	5.73
Latrine availability	No	96 (99.0)	1 (1.0)	97 (34.2)
	Yes	73 (39.0)	114 (61.0)	187 (65.8)
Water quality	Low quality	21 (100.0)	0 (.0)	21 (7.4)
	Medium quality	28 (100.0)	0 (.0)	28 (9.9)
	High quality	120 (51.1)	115 (48.9)	235 (82.7)
Socioeconomic status	Low	93 (98.9)	1 (1.1)	94 (33.1)
	Middle	61 (62.9)	36 (37.1)	97 (34.2)
	High	15 (16.1)	78 (83.9)	93 (32.7)

Table 1: Socio-demographic and economic characteristics of household having children aged 6-59 months stratified by household food insecurity status in Gambella Town, 2016.

We also assessed food allocation among household members. In 188 (66.2%) households, diets were first given for children even though the foods to be eaten were small during meal but its frequency is decreased with an increase in food insecurity level. In thirty five (12.3%) of households, foods were first given to husband and then shared among other family members. About near to one third, 91 (32.0%) children eat their diet after their father and mothers and also the percentage increase with across the food insecurity condition.

To look at the association between household food insecurity and nutritional status, bivariate analyses were done to identify candidate covariates for the multivariable model at p-value <0.25. Age of child, sex of child and household family size had p-value greater than 0.25 in bivariate analysis and thus, excluded from multivariable model. After identifying the candidate variables for multivariable analysis, we adjusted household food insecurity for all candidate covariates using multivariable logistic regression model to evaluate the association between household food insecurity and nutritional status of preschool children in Gambella Town. Accordingly, household food insecurity, duration of breast feeding for a child, household socioeconomic status, maternal educational status and access to quality of drinking water was significantly associated with stunting among study subject (Table 2). We also checked the interaction of household food allocation and household socioeconomic status with household food insecurity to look at whether the association between food insecurity and stunting is mediated by these two variables. No statistically significant interactions between food insecurity and these two variables were noted in our analyses. On bivariate analyses, wasting and underweight had *p*-value of greater than 0.25 and thus, their associations with household food insecurity were declared not significant (p=0.61 & 0.98, respectively). The findings were similar even after adjusting for potential confounders using multivariable regression models.

The present study showed that household food insecurity was found to be independent predictor significantly associated with stunting after controlling for possible confounders using multivariable analysis. Children from food insecure households were about 2.88 more likely to be stunted as compared to those children from food secure households. This association was more pronounced by the degree of severity of food insecurity; the odds of stunting was higher in children who were from severely and moderately food insecure households (AOR=9.78, 95% CI: 3.75-25.08 vs. AOR=2.92, 95% CI: 1.14-7.21, respectively).

Children who were living in households that had low quality of drinking water were 4.5 more likely to be stunted compared to children who were living in households with high quality of drinking water (AOR=4.5; 95% CI: 1.28-15.74). Those children who were breastfed for less than one year were 3 times more likely to be stunted compared

Characteristics		Nutritional status		COR (95% CI)	AOR (95% CI)
		Stunted	Normal		
		N (%)	N (%)		
Food security status*	Food insecure	57 (33.7)	112 (66.3)	5.99 (2.82, 12.70)	2.88 (1.26, 6.60)
	Food secure	9 (7.8)	106 (92.2)	Reference	Reference
Education (mother)	No formal education	49 (34.3)	94 (65.7)	3.80 (2.05, 7.02)	2.58 (1.27, 5.20)
	Formal education	17 (12.1)	124 (87.9)	Reference	Reference
Latrine	No	39 (40.2)	58 (59.8)	3.98 (2.24, 7.08)	1.45 (0.57, 3.67)
	Yes	27 (14.4)	160 (85.6)	Reference	Reference
Sick child feeding practice	Others	41 (39.8)	62 (60.2)	4.12 (2.31, 7.35)	2.32 (0.76, 7.06)
	Providing additional food	25 (13.8)	156 (86.2)	Reference	Reference
Breast feeding duration	<1year	45 (37.8)	74 (62.2)	4.17 (2.31, 7.51)	3.0 (1.56, 5.78)
	≥1year	21 (12.7)	144 (87.3)	Reference	Reference
Child seek health	After 24 h	34 (41.5)	48 (58.5)	3.76 (2.10, 6.71)	0.94 (0.30, 2.96)
	Within 24 h	32 (15.8)	170 (84.2)	Reference	Reference
Marital status (mother)	Not married	50 (19.9)	201 (80.1)	5.65 (2.86, 11.17)	1.89 (0.52, 6.82)
	Married/living married	16 (48.5)	17 (51.5)	Reference	Reference
Drinking water quality	Low quality	14 (66.7)	7 (33.3)	10.05 (3.81, 26.52)	4.51 (1.28, 15.74)
	Medium quality	13 (46.4)	15 (53.6)	4.35 (1.92, 9.87)	2.31 (0.95, 5.59)
	High quality	39 (16.6)	196 (83.4)	Reference	Reference
Food insecurity levels*	Food secure	9 (7.8)	106 (92.2)	Reference	Reference
	Mildly food insecure	10 (17.5)	47 (82.5)	2.50 (0.95, 6.57)	1.81 (0.66, 4.97)
	Moderately food insecure	21 (31.3)	46 (68.7)	5.37 (2.28, 12.63)	2.92 (1.17, 7.30)
	severely food insecure	26 (57.8)	19 (42.2)	16.11 (6.54, 39.70)	9.78 (3.80, 25.15)
Wealth Index	Low	43 (45.7)	51 (54.3)	10.35 (4.33, 24.74)	4.24 (1.48, 12.09)
	Middle	16 (16.5)	81 (83.5)	2.42 (0.94, 6.20)	2.11 (0.78, 5.65)
	High	7 (7.5)	89 (92.5)	Reference	Reference

*Estimation of parameters was done by entering both forms of the variable into the model separately to avoid collinearity between them; COR: Crude Odds Ratio; AOR: Adjusted Odds Ratio; CI: Confidence Interval

Table 2: Associations between stunting and selected variables among children aged 6-59 months in Gambella Town, 2016.

to those children who were breastfed for one year and above (AOR=3; 95% CI=1.56-5.78).

In addition, low socio economic status of households was significantly associated with child stunting. The odds of being stunted among children from low socioeconomic status was 4.2 times higher compared to those children from high socioeconomic status households (AOR=4.2; 95% CI=1.48-12.09). Further, educational status of mothers was significantly associated with stunting in this study; children from mothers who had no formal education were 2.6 more likely to be stunted compared to their counterparts (AOR=4.6; 5% CI=1.27-5.20).

Discussion

This study revealed that 23.2%, 12.0% and 13.4% of children were stunted, underweight and wasted, respectively. Only 3.5% of children were overweight. The current findings are lower than the findings from 2011 EDHS for Gambella region showing stunting, wasting, and underweight were 27.3%, 12.5%, and 20.7%, respectively [19]. On the other hand, these findings are somewhat similar to the findings from Mini EDHS 2014 for Gambella region where stunting, wasting; underweight were 22.5%, 14.9%, and 18.5%, respectively [14]. However, the overweight/obesity prevalence of 3.5% was higher than the findings from both 2011 EDHS and 2014 Mini EDHS findings in the Gambella region (0.7% and 0.4%, respectively). The higher and lower prevalence of overweight/obesity and underweight in this study as compared to the findings from EDHS could be explained by the differences in study setting as the EDHS findings included rural population while this study was based on urban population alone. Evidence shows, rapid demographic, social and economic changes ongoing in many developing countries have led to increased urbanization and changes in food systems resulting in a global nutrition transition where recent global shifts in dietary patterns towards higher intakes of saturated fats, sugars and refined foods, and lower intakes of fiber rich foods, driven by technological advances that have made energy dense, nutrient-poor foods cheaply available on global food markets which could have increased overweight/obesity and reduced underweights [20]. In this global context, while large inequalities from the burden of under nutrition persist across regions, countries and communities, a concomitant increase in rates of overweight and obesity is witnessed, often in these same communities resulting in double burden [21].

In this study household food insecurity was significantly associated with stunting where children from food insecure household in general and those severely and moderately food insecure households in particular had higher odds of being stunted. Based on these findings, it can be inferred that children's nutritional status in Gambella town is significantly associated with both household socioeconomic status and food security. Any situation that limits real incomes of families and in the accessibility of food can be expected to result in a substantial faltered growth and hence, could end in stunting. As this study was conducted in the urban setting where most households procure their food supplies through purchases, it is thus understandable that households which produce a major share of the food they consume may be less subject to insecurity than households which depend almost entirely on purchased foods. This perhaps partly explains the close links of food insecurity and stunting among children in the study area. Other studies have reported that household level poverty rather than food insecurity is predictive of malnutrition among children [22]. Although our study did not measure

poverty directly, the significant associations between wealth index and child stunting, suggests that poverty may be a major determining factor of nutritional status of children in Gambella Town.

The result of the present study is similar with that of study carried out in Tigray region of Ethiopia that shows there was statistically significant difference in stunting between food secure and food insecure households in which children from food insecure households had about 48% at higher odds to be stunted when compared to the children of food secure households [23]. Moreover, similar findings were reported from a cross-sectional study conducted on less than 5 years children from Bangladesh, Ethiopia and Vietnam where the odds of being stunted were significantly higher for children in severely food-insecure households in Bangladesh and Ethiopia while the higher odds of being stunted was higher for children in moderately food-insecure households in Vietnam [9]. In general, many findings from cross-sectional studies showed that there were significant associations between household food insecurity and adverse child growth outcomes [22,24-27]. These finding are further supported by findings from a longitudinal study on children less than 5 years from Ethiopia, India and Vietnam where children from food-insecure households had significantly lower HAZs in all countries at 5 year of follow up [28]. However, few studies show that there were no significant associations between food insecurity and stunting among preschool children [1,5].

Stunting was also significantly associated with household socioeconomic status in multivariable analysis. Children from households found in the low level of socio-economic status had increased odds of being stunted compared to those found in high socioeconomic status households. The finding from this study is similar to the finding reported by other studies in Holetta district, Oromiya region in central Ethiopia where child malnutrition (measured by stunting) is significantly lower in households with crossbred cows (i.e., better quality) than in those without [29]. In addition, there is evidence showing low household income negatively affects household food security that might have been linked with stunting [30].

The other variable that was found to be independently associated with stunting in this study was maternal educational status. Children from mothers/caregivers who had no formal education had significantly higher odds to be stunted compared to their counterparts. This finding is supported by findings from other studies conducted by Christiaensen and Alderman [29], which showed the effect of maternal education, is about twice as important as that of paternal education. Moreover, this study demonstrated that presence of at least one adult female completing primary school in a household resulted in a 6-11 percent decline in stunting, while completion of primary school by at least one male adult reduces child stunting by only 2-8 percent [29]. Further, food insecurity was negatively associated with education level mothers and fathers of Iranian children under the study that might contribute to the prevalent malnutrition in their children [30].

Furthermore, duration of breast-feeding beyond 12 months is significantly associated with child stunting. Evidences show that the early child detachment from breast-feeding significantly affects the health and nutritional status of the child through reduction of care and exposing the child to early weaning, which in turn increases the risk of diarrheal diseases and nutritional deficiencies [9]. Supporting this fact, the present study revealed that the longer the duration of breast feeding, the lesser the odds of being stunted. Similarly study undertaken in the Tigray region showed children who were breastfed for the longer duration had less odds to be stunted [23].

The current study demonstrated that household quality of drinking water was significantly associated with stunting in children where children from households with low quality of drinking water had higher odds of being stunted. This finding was supported by other study result that water and sanitation play a particularly important role in child nutrition due to their impact on diarrheal diseases and consecutive loss of appetite and growth faltering [31].

In this study, underweight and wasting were not associated with household food insecurity. This result was not expected, because of the substantial evidence that a household's access to food are among the key determinants of nutritional status of children. Nevertheless, this lack of association might be explained by several factors. In line with our analytical pathways, many studies suggest that the influence of food supply and access on nutritional status of children can be confounded by other key determinants of child nutrition, such as maternal knowledge on child nutrition and caring practices, maternal nutritional status, intra household food allocation and utilization, access to health services, and healthful environment like hygiene and sanitation [24]. However, Amaha et al., [23] reported similar finding where underweight and wasting in children had no association with household food insecurity. Other researchers also found that food insecurity had no association with wasting.

Our study has some limitations. Because of the cross-sectional nature of the study, it is impossible to establish a temporal relationship and thus, causality between food security status and nutritional status of study subjects. In addition, some important variables such as maternal nutritional status or knowledge of child nutrition, morbidity among children and dietary intake that may also contribute to the nutritional outcomes of interest were not included in this study. A further, seasonal variation in food security status that could influence the nutritional status of children was not investigated in the present study. However, our study has a number of strengths. The sampling procedure and community based natures of our study can be considered as strength for our study as these could allow us to infer our results to the general population under the study.

Conclusion

This study demonstrated that stunting, underweight and wasting were prevalent among preschool children in Gambella Town. In this study, household food insecurity was significantly associated with stunting, but not with underweight and wasting among children studied. The study also revealed emerging prevalence of overweight and/or obesity among study subjects. The finding implies nutrition interventions targeting children need to address household food security.

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Author Contributions

ZJ conceived the idea; ZJ, KH and TW designed the study; ZJ supervised data collection, ZJ and TW analyzed and interpreted the data. ZJ and TW drafted the manuscript and reviewed the drafted manuscript critically. All authors gave final approval of the paper to be published.

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Classification of Dual Burden of Malnutrition in Young Children

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Abstract

Introduction: Available classifications and indices for assessment of malnutrition are limited to deficiencies. Societies with transitional nutrition have children suffering not only deficiencies, but also excess or both (dual burden). This paper proposes a classification of nutritional status for identifying normal and failure conditions due to deficiency, excess and dual burden as well as of their components.

Methods: One nutritional condition was considered normal and 12 malnutrition conditions were used for the proposed Malnutrition Anthropometric Index (MAI) as the summation of malnutrition conditions shares and comprising sub-indices of deficiencies (MAD), of excess (MAE) or dual burden (MADB). These indices and 95% CI for comparisons were computed utilizing data from 426 children from six to 59 months of age derived from household surveys of 14 rural bordering communities from Central America in 2013-2015, 9144 sampled national Guatemalan children, 3311 urban and 5833 rural in 2008-2009, and 5892 sampled national Burkina Faso children, 1311 urban and 4581 rural in 2010.

Results: Shares and 95% confidence intervals estimated for proposed indices were able to differentiate dual burden as well as other indices among study populations.

Conclusions: MAI was better estimate of malnutrition and sub-indices MAD, MAE and MADB and their components provided breakdown of malnutrition conditions as inputs for a better understanding of malnutrition and better decision-making.

Recommendations: Consider malnutrition conditions due to deficiencies as well as excess and dual burden in nutritional assessments of children. Use MAI as well as sub-indices MAD, MAE and MADB and their components in the analysis of results from anthropometric surveys in children for monitoring and evaluation purposes in actions and policies with impact on food and nutrition security.

Keywords: Double burden of malnutrition in young children; Malnutrition index; Nutritional status classification

Introduction

Current classifications and indices for assessing nutritional status in children are limited to malnutrition due to deficiencies [1-4]. In this sense these classifications are insufficient for guiding food and nutrition security public policies based on children malnutrition due to deficiencies, excess or both (dual burden). These indices of anthropometric failure consider categories of three standard Z scores and under-estimate malnutrition due to deficiencies because of the exclusion of malnutrition conditions due to excess as well as dual burden and consequently over-estimate the proportion of children in normal nutrition conditions.

Several studies in Central America, for example in Guatemala, have highlighted the occurrence of growth retardation (stunting) in early ages and by the fifth year, one half of children were stunted [5] and remained stunted by the ninth year of age [6]. Other studies in urban children of public school in Guatemala City found that almost one tenth children were stunted and of this proportion one fifth of girls and one fourth of boys were also obese [7]. This is dual burden of malnutrition which cannot be assessed using the available indices of anthropometric failure based on malnutrition due to deficiencies only.

On the other hand the country reports in the WHO database (WHO [8]) are based on separate standardized Z scores of height for age (HAZ), weight for height (WHZ) and body mass index (BMIZ). The percentages of children suffering stunting, wasting and obesity do not allow assessing dual burden of malnutrition. The estimates of stunting (HAZ<-2), wasting (WHZ or BMIZ<-2) and obesity (WHZ or BMIZ ≥ +2) were derived from surveys or censuses carried out by countries.

The problem of nutritional assessment reports of young children is that with transitional nutritional status of societies we do not know how much of stunting children are growing properly and how much are not, in particular malnourished due to deficiency such as wasting and malnourished due to excess such as overweight or obesity. These estimates do not provide inputs for estimating the dual burden of malnutrition.

The proposed classification of nutritional anthropometry status aims to estimate the proportion of acceptable normal nutritional condition as well as the proportions of nutritional failure conditions which are due to deficiency, excess and both (dual burden) and their components for providing inputs to guidelines for actions of public food and nutrition policies. The proportions of components for each of the three nutritional failure conditions (deficiency, excess and both or

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dual burden) help to understand the nature of malnutrition and the call for policy and actions in the short as well as medium and long-terms.

Methods

Table 1 describes in the last five columns to the right the 18 possible combinations of 2, 3 and 3 categories of the standardized nutritional anthropometric indexes of height for age (HAZ), body mass index (BMIZ) and weight for age (WAZ) depicting nutritional conditions. The HAZ two categories are stunting (HAZ<-2) and normal growth (HAZ ≥ -2), the BMIZ three categories are wasting (BMIZ<-2), normal nutrition (-2 ≤ BMIZ<+2) and overweight or obesity (BMIZ ≥ +2), and the WAZ three categories are low weight for age (WAZ<-2), normal weight for age (-2 ≤ WAZ<+2) and high weight for age (WAZ ≥ +2). Out of 18 possible combinations of HAZ, BMIZ and WAZ categories, 13 are considered feasible (one for normal, six for deficiencies, three for dual burden and three for excess) and five non-viable in the last five rows.

Column two describes categories of the nutritional status classification by Waterlow et al. [1] and column three Nandy et al. [3] categories. It is clear in Table 1 that Waterlow et al. [1] proposal comprised in the action? category in column two, all dual burden of malnutrition conditions and also malnutrition conditions due to deficiency such as stunting only as well as stunting with low weight for age; this probably was the reason for giving a questioning name to this category and the kind of action for stunted children. In addition, Waterlow et al. [1] did not include in any category of malnutrition conditions due to excess at all. The normal category was over-estimated.

Svedberg [2] and Nandy et al. [3] identified malnutrition conditions due to deficiency with letters B, C, D, E and F. However, it can be seen in Table 1 that categories E and F included dual burden of malnutrition conditions, so that it cannot be separated from malnutrition due to deficiency. In addition, they did not include any category of malnutrition conditions due to excess at all and the normal category was over-estimated, as in the case of Waterlow et al. [9].

The first row of categories corresponds to children in normal nutritional status, that is, with no malnutrition conditions of stunting (ST), wasting (WS), overweight or obesity (OW), low weight for age (LW) or high weight for age (HW) as shown in the last five columns.

The rows from two to seven of nutritional status categories correspond to children in malnutrition due to deficiency (MAD), that is, WS, WSLW, STWSLW, STLW, ST and LW; from eight to ten to children in malnutrition due to dual burden (MADB), that is, STOWLW, STOW and STOWHW; and from eleven to thirteen to children in malnutrition due to excess (MAE), that is, HW, OW and OWHW.

The proposed Malnutrition Anthropometric Index (MAI) is the summation of the proportions of all 12 malnutrition categories, that is, WS, WSLW, STWSLW, STLW, ST, LW, STOWLW, STOW, STOWHW, HW, OW and OWHW. The sub-indices MAD, MADB and MAE are the summations of their corresponding proportions of malnutrition categories. It is expected that the structures of MAI, MAD, MAE and MADB differ from population to population linked to dietary causal factors and other non-dietary factors and their interactions.

We found that conceptually five combinations are non-viable

Condition	Waterlow categories	Nandy et al. categories	Nutritional status categories	ST	WS	OW	LW	HW
Normal	Normal	A	NN	NO	NO	NO	NO	NO
Malnutrition Anthropometric Index (MAI)	-	-	-	-	-	-	-	-
Malnutrition due to Deficiency (MAD)	-	-	-	-	-	-	-	-
Wasting only (WS)	Action	B	WS	NO	YES	NO	NO	NO
Wasting (WS) with low weight for age (LW)	Action	C	WSLW	NO	YES	NO	YES	NO
Wasting (WS) with stunting (ST) and low weight for age (LW)	Priority	D	STWSLW	YES	YES	NO	YES	NO
Stunting (ST) with low weight for age (LW)	Action?	E	STLW	YES	NO	NO	YES	NO
Stunting only (ST)	Action?	F	ST	YES	NO	NO	NO	NO
Low weight for age only (LW)	-	Y	LW	NO	NO	NO	YES	NO
Malnutrition due to Dual Burden (MADB)	-	-	-	-	-	-	-	-
Stunting (ST) with overweight or obesity (OW) and low weight for age (LW)	Action?	E	STOWLW	YES	NO	YES	YES	NO
Stunting (ST) with overweight or obesity (OW)	Action?	F	STOW	YES	NO	YES	NO	NO
Stunting (ST) with overweight or obesity (OW) and high weight for age (HW)	Action?	F	STOWHW	YES	NO	YES	NO	YES
Malnutrition due to Excess (MAE)	-	-	-	-	-	-	-	-
High weight for age only (HW)	-	-	HW	NO	NO	NO	NO	YES
Overweight or obesity (OW) only	-	-	OW	NO	NO	YES	NO	NO
Overweight or obesity (OW) with high weigh for age (HW)	-	-	OWHW	NO	NO	YES	NO	YES
Non viable conditions	-	-						
Wasting (WS) with stunting (ST)	Priority	-	STWS	YES	YES	NO	NO	NO
Wasting (WS) with stunting (ST) and high weight for age (HW)	Priority	-	STWSHW	YES	YES	NO	NO	YES
Stunting (ST) with high weight for age (HW)	Action?	-	STHW	YES	NO	NO	NO	YES
Wasting (WS) with high weight for age (HW)	Action	-	WSHW	NO	YES	NO	NO	YES
Overweight or obesity (OW) with low weight for age (LW)	-	-	OWLW	NO	NO	YES	YES	NO

Table 1: Classification of the nutritional status of children.

conditions in the sense of plausibility. Overweight or obesity with low weight for age (OWLW) is contradictory.

The proposed classification and the estimations of index MAI, sub-indices MAD, MAE and MADB as well their 12 malnutrition conditions were illustrated by utilizing the following data: 426 children from six to 59 months of age from 14 bordering communities from rural Central America (RCA) in 2013-2015; 9144 sampled national Guatemalan children, 3311 urban (UGT) and 5833 rural (RGT) in 2008-2009; and 5892 children from Burkina Faso, 1311 urban (UBF) and 4581 rural (RBF) in 2010.

The RCA dataset includes anthropometric data collected in household surveys carried out in small rural villages in bordering municipalities of El Salvador, Guatemala, Honduras and Nicaragua by the Regional Programme of Food and Nutrition Security for Central America (PRESANCA II in Spanish) and the Regional Programme of Information Systems on Food and Nutrition Security (PRESISAN in Spanish), both of the General Secretariat of the Central American Integration System (SG-SICA in Spanish). These surveys were carried out in 2013, 2014 and 2015 as part of the baseline for measuring food and nutrition food insecurity [10].

The UGT, RGT, UBF and RBF national level survey datasets were downloaded from data repositories. The Guatemala 2008-2009 dataset is available at the Global Health Data Exchange [11] and Burkina Faso datasets at Measuredhs [12]. Demographic and Health Surveys (DHS) of the DHS Program supported by United States Agency for International Development (USAID).

The datasets were processed using software WHO ANTHRO PLUS with 2006 WHO growth patterns for computing HAZ, BMIZ and WAZ indices with valid measures of weight, height or length for age and sex. Measures of weight, height or length were obtained by trained and standardized personnel for the RCA study, while for the DHS survey data we assumed were standardized as required by DHS protocols. Recoding of HAZ, BMIZ and WAZ as well as proportion and confidence limits were estimated using software Epi-Info [13].

Results

Tables 2-6 report on RCA, UGT, RGT, UBF and RBF results respectively. The results are expressed in terms of number of children, estimates of proportions and 95% confidence limits. Note that RCA data for several villages were censuses of households, while in UGT, RGT, UBF and RBF were samples with sampling designs different than simple random samples. However we have assumed a simple random sampling for easiness of the illustrations.

For RCA (Table 2) only results for MAI, MAD, MADB and MAE are presented due to small number of children in their categories.

Figure 1 shows nutritional status classification for children in rural Central America the MAI was 51.2% comprising a MAD of 43.7%, a MAE of 4.0% and a MADB of 3.5%.

In urban Guatemala the MAI was 42.9% with MAD of 36.8%, MAE of 4.2%, and MADB of 2.0%. In contrast, rural Guatemala the MAI was higher by close to 20% points (63.2%), mainly due to a higher MAD (57.6%) as shown in Tables 3 and 4. The MAE in rural Guatemala was lower with 2.1% (95% CI from 1.7% to 2.5%) than in urban Guatemala with 4.2% (95% CI from 3.5% to 4.9%), while the opposite for MADB, rural Guatemala was higher with 3.5% (95% CI from 3.1% to 4.0%)

Condition	Nutritional status category	Number of children	Proportion (%)	Lower CL	Upper CL
Normal	NN	208	48.8	44	53.7
Malnutrition Anthropometric Index (MAI)	-	218	51.2	46.3	56
Malnutrition due to Deficiency (MAD)	-	186	43.7	38.9	48.5
Malnutrition due to Dual Burden (MADB)	-	15	3.5	2.1	5.9
Malnutrition due to Excess (MAE)	-	17	4	2.4	6.4

Table 2: Proportions and confidence limits (95%) of nutritional status categories in RCA children.

Condition	Nutritional status category	Number of children	Proportion (%)	Lower CL	Upper CL
Normal	NN	1891	57.1	55.4	58.8
Malnutrition Anthropometric Index (MAI)	-	1420	42.9	41.2	44.6
Malnutrition due to Deficiency (MAD)	-	1217	36.8	35.1	38.4
Wasting only (WS)	WS	4	0.1	0	0.3
Wasting (WS) with low weight for age (LW)	WSLW	5	0.2	0.1	0.4
Wasting (WS) with stunting (ST) and low weight for age (LW)	STWSLW	5	0.2	0.1	0.4
Stunting (ST) with low weight for age (LW)	STLW	288	8.7	7.8	9.7
Stunting only (ST)	ST	907	27.4	25.9	29
Low weight for age only (LW)	LW	8	0.2	0.1	0.5
Malnutrition due to Dual Burden (MADB)	-	65	2	1.5	2.5
Stunting (ST) with overweight or obesity (OW) and low weight for age (LW)	STOWLW	0	0	0	0.1
Stunting (ST) with overweight or obesity (OW)	STOW	65	2	1.5	2.5
Stunting (ST) with overweight or obesity (OW) and high weight for age (HW)	STOWHW	0	0	0	0.1
Malnutrition due to Excess (MAE)	-	138	4.2	3.5	4.9
High weight for age only (HW)	HW	6	0.2	0.1	0.4
Overweight or obesity only (OW)	OW	98	3	2.4	3.6
Overweight or obesity (OW) with high weigh for age (HW)	OWHW	34	1	0.7	1.4

Table 3: Proportions and confidence limits (95%) of nutritional status categories in UGT children.

Condition	Nutritional status category	Number of children	Proportion (%)	Lower CL	Upper CL
Normal	NN	2146	36.8	35.6	38
Malnutrition Anthropometric Index (MAI)	-	3687	63.2	62	64.4
Malnutrition due to Deficiency (MAD)	-	3361	57.6	56.3	58.9
Wasting only (WS)	WS	9	0.2	0.1	0.3
Wasting (WS) with low weight for age (LW)	WSLW	16	0.3	0.2	0.5
Wasting (WS) with stunting (ST) and low weight for age (LW)	STWSLW	18	0.3	0.2	0.5
Stunting (ST) with low weight for age (LW)	STLW	952	16.3	15.4	17.3
Stunting only (ST)	ST	2342	40.2	38.9	41.4
Low weight for age only (LW)	LW	24	0.4	0.3	0.6
Malnutrition due to Dual Burden (MADB)	-	206	3.5	3.1	4
Stunting (ST) with overweight or obesity (OW) and low weight for age (LW)	STOWLW	7	0.1	0.1	0.3
Stunting (ST) with overweight or obesity (OW)	STOW	199	3.4	3	3.9
Stunting (ST) with overweight or obesity (OW) and high weight for age (HW)	STOWHW	0	0	0	0.1
Malnutrition due to Excess (MAE)		120	2.1	1.7	2.5
High weight for age only (HW)	HW	2	0	0	0.1
Overweight or obesity only (OW)	OW	91	1.6	1.3	1.9
Overweight or obesity (OW) with high weigh for age (HW)	OWHW	27	0.5	0.3	0.7

Table 4: Proportions and confidence limits (95%) of nutritional status categories in RGT children.

Condition	Nutritional status category	Number of children	Proportion (%)	Lower CL	Upper CL
Normal	NN	810	61.8	59.1	64.4
Malnutrition Anthropometric Index (MAI)	-	501	38.2	35.6	40.9
Malnutrition due to Deficiency (MAD)	-	450	34.3	31.8	37
Wasting only (WS)	WS	47	3.6	2.7	4.8
Wasting (WS) with low weight for age (LW)	WSLW	78	5.9	4.8	7.4
Wasting (WS) with stunting (ST) and low weight for age (LW)	STWSLW	35	2.7	1.9	3.7
Stunting (ST) with low weight for age (LW)	STLW	124	9.5	8	11.2
Stunting only (ST)	ST	146	11.1	9.5	13
Low weight for age only (LW)	LW	20	1.5	1	2.4
Malnutrition due to Dual Burden (MADB)	-	33	2.5	1.8	3.6
Stunting (ST) with overweight or obesity (OW) and low weight for age (LW)	STOWLW	0	0	0	0.4
Stunting (ST) with overweight or obesity (OW)	STOW	33	2.5	1.8	3.6
Stunting (ST) with overweight or obesity (OW) and high weight for age (HW)	STOWHW	0	0	0	0.4
Malnutrition due to Excess (MAE)	-	18	1.4	0.8	2.2
High weight for age only (HW)	HW	2	0.2	0	0.6
Overweight or obesity only (OW)	OW	12	0.9	0.5	1.6
Overweight or obesity (OW) with high weigh for age (HW)	OWHW	4	0.3	0.1	0.8

Table 5: Proportions and confidence limits (95%) of nutritional status categories in UBF children.

versus 2.0% (95% CI from 1.5% to 2.5%) in urban Guatemala as depicted by Tables 3 and 4.

The magnitude of malnutrition in Burkina Faso was slightly lower in urban children and lower in rural children than in Guatemala. The urban-rural gap was higher in Guatemala than in Burkina Faso.

In urban Burkina Faso the MAI was 38.2% with a MAD of 34.3%, a MAE of 1.4% and a MADB of 2.5%; while in rural Burkina Faso the MAI was circa 15% points higher (53.8%) with a MAD of 49.9%, and MAE and MADB of the same magnitude respectively.

Surprisingly children living in remote rural villages of bordering municipalities from El Salvador, Guatemala, Honduras and Nicaragua in 20013-2015 showed higher normal nutritional conditions with prevalence of 48.8% and 95% confidence interval from 44.0% to 53.7% (Table 2) than those living in rural areas of Guatemala 2008-2009 with prevalence of 36.8% and 95% confidence interval from 35.6% to 38.0% (Table 4).

Malnutrition conditions due to excess were higher in Central America populations (RCA, UGT and RGT) than in Burkina Faso; however malnutrition conditions due to dual burden were similar. On the other hand, malnutrition due to dual burden and excess were similar in urban and rural children in Burkina Faso.

Figures 2-6 show details of structural composition of the sub-indices of malnutrition (MAD, MAE and MADB). Figures 2-4 show predominant MAD components in RCA of three fourths (31.9%) due

Condition	Nutritional status category	Number of children	Proportion (%)	Lower CL	Upper CL
Normal	NN	2115	46.2	44.7	47.6
Malnutrition Anthropometric Index (MAI)	-	2466	53.8	52.4	55.3
Malnutrition due to Deficiency (MAD)	-	2286	49.9	48.4	51.4
Wasting only (WS)	WS	207	4.5	3.9	5.2
Wasting (WS) with low weight for age (LW)	WSLW	246	5.4	4.7	6.1
Wasting (WS) with stunting (ST) and low weight for age (LW)	STWSLW	174	3.8	3.3	4.4
Stunting (ST) with low weight for age (LW)	STLW	772	16.9	15.8	18
Stunting only (ST)	ST	800	17.5	16.4	18.6
Low weight for age only (LW)	LW	87	1.9	1.5	2.3
Malnutrition due to Dual Burden (MADB)	-	115	2.5	2.1	3
Stunting (ST) with overweight or obesity (OW) and low weight for age (LW)	STOWLW	8	0.2	0.1	0.4
Stunting (ST) with overweight or obesity (OW)	STOW	107	2.3	1.9	2.8
Stunting (ST) with overweight or obesity (OW) and high weight for age (HW)	STOWHW	0	0	0	0.4
Malnutrition due to Excess (MAE)		65	1.4	1.1	1.8
High weight for age only (HW)	HW	8	0.2	0.1	0.4
Overweight or obesity only (OW)	OW	34	0.7	0.5	1
Overweight or obesity (OW) with high weigh for age (HW)	OWHW	23	0.5	0.3	0.8

Table 6: Proportions and confidence limits (95%) of nutritional status categories in RBF children.

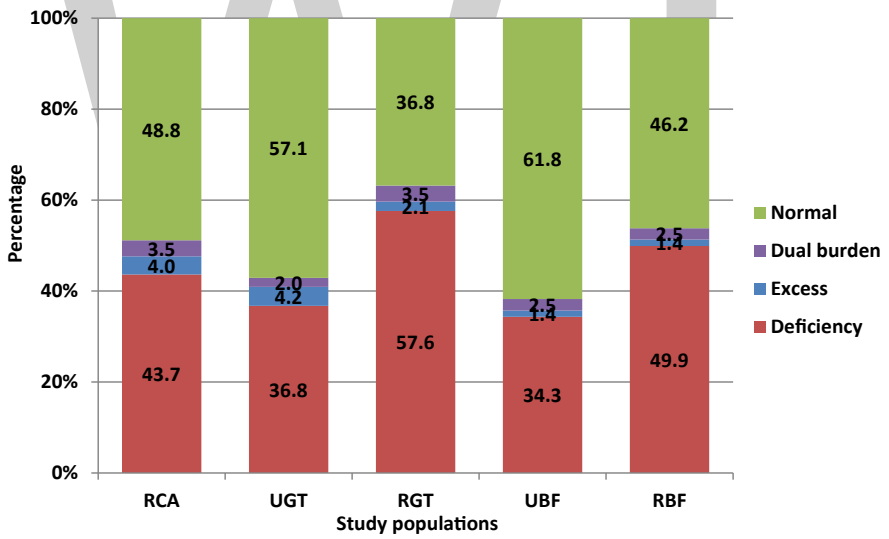


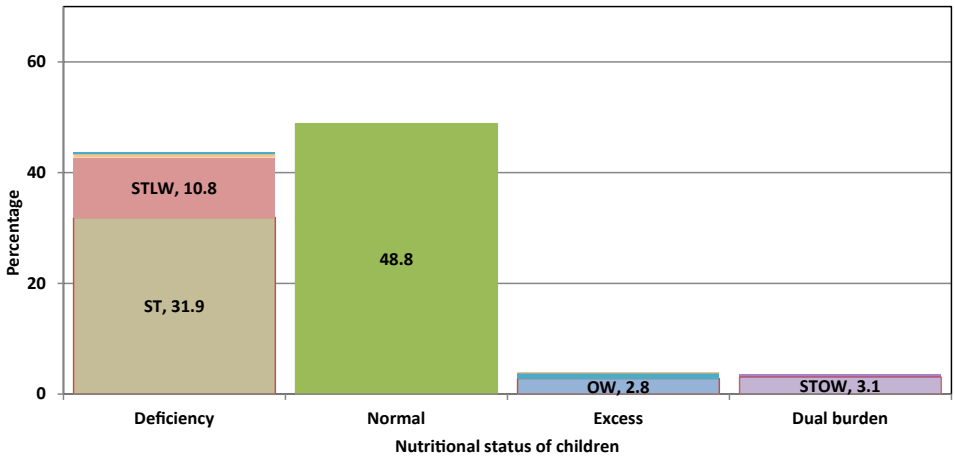
Figure 1: Classification of the nutritional status of children in rural Central America 2013-2015 (RCA), urban and rural Guatemala 2008-2009 (UGT and RGT respectively) and rural and urban Burkina Faso 2010 (UBF and RBF respectively).

to stunting alone and one fourth (10.8%) to stunting with low weight for age, in urban Guatemala three fourths (27.4%) due to stunting alone and one fourth (8.7%) to stunting with low weight for age, the MAE was 4.2%, mainly due to overweight or obesity alone (3.0%) and the MADB was 2.0%, mainly due to stunting and overweight or obesity (2.0%); and in rural Guatemala three fourths (40.2%) due to stunting alone and one fourth (16.3%) to stunting with low weight for age; the MAE was 2.1%, mainly due to overweight or obesity alone (1.6%) and the MADB was 3.5%, mainly due to stunting and overweight or obesity (3.4%).

Both MAE and MADB may become a public nutrition problem in Central America, in particular overweight or obesity alone or with stunting. The MAE were 4.0%, 4.2% and 2.1% in RCA, UGT and RGT respectively, mainly due to overweight or obesity alone (2.8%, 3.0% and 1.6% respectively); while the MADB were 3.5%, 2.0% and 3.5% respectively, mainly due to stunting with overweight or obesity (3.1%, 2.0% and 3.4% respectively).

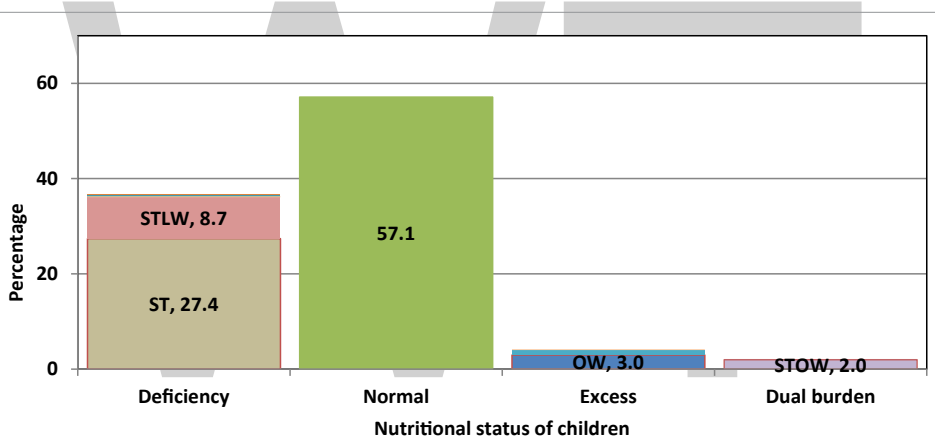
Figures 5-6 show that the structural compositions for children in rural and urban Burkina Faso 2010 were similar but very different from those study populations in Central America. Figure 5 shows that the MAD structural composition of deficiency conditions was less predominant and complex than that observed in Central America populations.

For children in urban Burkina Faso the MAD was comprised by many malnutrition conditions. Stunting alone (11.1%), stunting with low weight for age (9.5%), wasting with low weight for age (6.0%) and wasting alone (3.6%) among others, and in rural children higher with stunting alone (17.5%), stunting with low weight for age (16.9%),



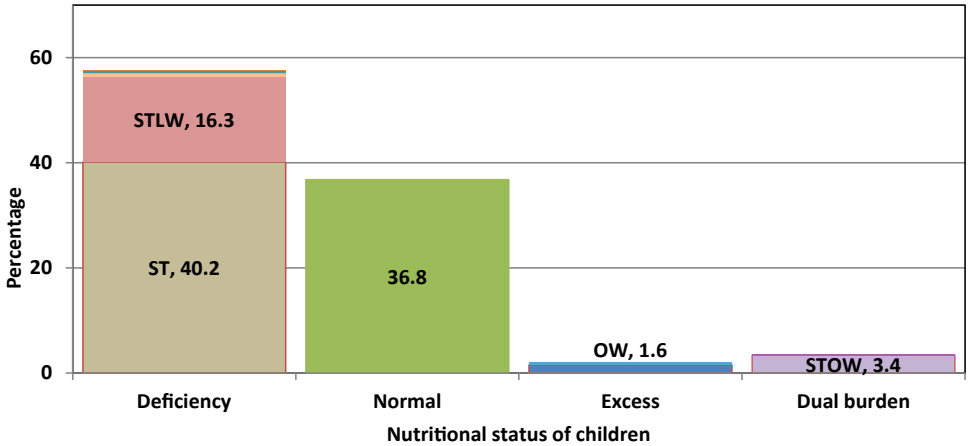
Codes of categories for nutritional status of HAZ, BMIZ and WAZ:
ST stunting, WS wasting, LW low weight for age, NN normal, OW overweight or obesity, HW high weight for age

Figure 2: Classification of the nutritional status of children in rural communities of bordering territories in El Salvador, Guatemala, Honduras and Nicaragua 2013-2015.



Codes of categories for nutritional status of HAZ, BMIZ and WAZ:
ST stunting, WS wasting, LW low weight for age, NN normal, OW overweight or obesity, HW high weight for age

Figure 3: Classification of the nutritional status of children in urban Guatemala 2008-2009.



Codes of categories for nutritional status of HAZ, BMIZ and WAZ:
ST stunting, WS wasting, LW low weight for age, NN normal, OW overweight or obesity, HW high weight for age

Figure 4: Classification of the nutritional status of children in rural Guatemala 2008-2009.

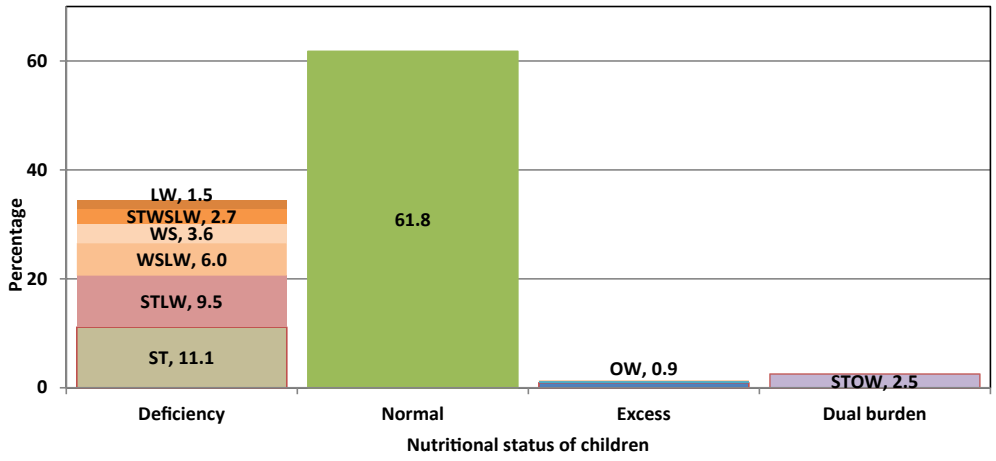


Figure 5: Classification of the nutritional status of children in urban Burkina Faso 2010.

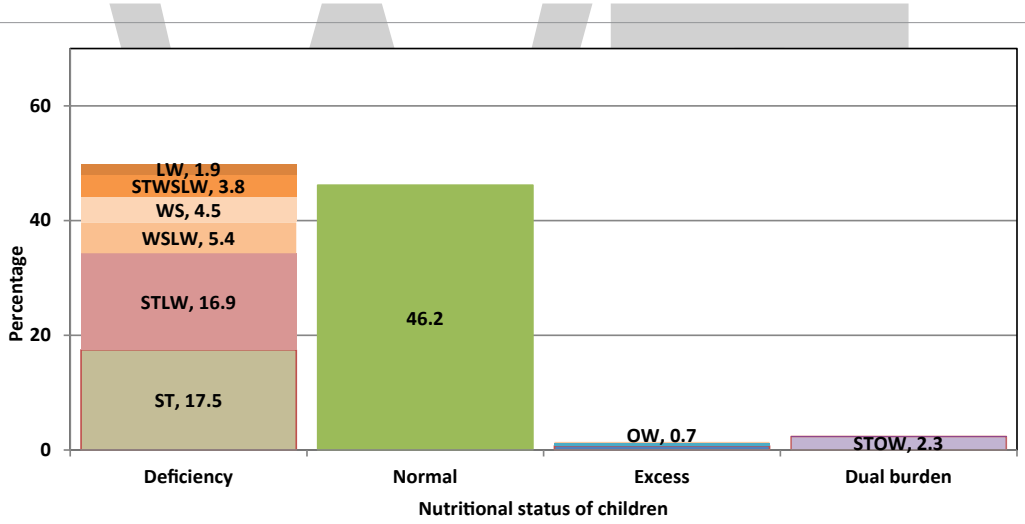


Figure 6: Classification of the nutritional status of children in rural Burkina Faso 2010.

wasting with low weight for their age (5.4%), and wasting alone (4.5%) among others.

The MAE and MADB were lower than in Central America study populations. The MAE was in urban and rural children mainly due to overweight or obesity alone (0.9% and 0.7% respectively) and the MADB due to stunting with overweight or obesity (2.5% and 2.3% respectively).

Conclusions

Malnutrition due to deficiencies as well as excess and dual burden should be assessed for a better guide to policy designers and decision makers of strategies, programmes, projects and actions with impact on the food and nutrition security of populations.

Policy-design implications for urban and rural populations may be different depending on malnutrition conditions due to deficiencies, to families of children facing dual burden stunting and overweight or obesity, and to families of children facing stunting alone to avoid overweight or obesity.

Discussion

Although we have limited our analysis to a small number of study populations for illustration purposes, this type of analysis is very useful for untangling the malnutrition assessment into deficiency, excess and dual burden in children based on traditional standardized Z scores of anthropometric indices and without adding collected information in the household surveys. For example, highlighting that urban children with higher MAE than rural children and the opposite with respect to MADB provides better knowledge for action in Guatemala.

Using the proposed approach the percentage of children in normal nutrition may be better estimated by considering malnutrition due to excess and dual burden, in particular in developing societies.

The existence of not only malnutrition due to excess but also dual

burden of malnutrition requires special attention in the kind of policies and actions needed to address emerging malnutrition problems.

Recommendations

Expand country reports in the WHO database (WHO, 2016) at including the percentages of children suffering deficiencies, excess and both using the sub-indices MAD, MAE and MADB.

Use MAI, sub-indices MAD, MAE and MADB as well as their components in the analysis of results from anthropometric surveys in children for monitoring and evaluation purposes in actions and policies with impact on food and nutrition security.

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Review of Nutraceuticals and Functional Properties of Whole Wheat

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Abstract

Wheat (*Triticum aestivum* L.) is one of the most commonly cultivated and consumed cereals throughout the world. Though phytochemicals and antioxidants in the cereal grains have not been studied as in fruits and vegetables, given the role of wheat in our diet plate, it is a given of primary importance to understand the chemistry of our major food, wheat. The presence of diverse polyphenols and their action against leading cause of death, including heart diseases, cancer, obesity, and diabetes, widens the scope of wheat. Phytochemicals such as phenolic acids, alkylresorcinols, flavonoids, phytosterols, and carotenoids are present in whole wheat. The majority of phytochemicals are located in the wheat bran/germ fraction, and they are the leading contributors to the health promoting activities. However, the presence of anti-nutrients and binding of phenolic acids with protein may have adverse effect on health. This review mainly focuses on studies that have been carried out in the past decade to present, emphasizing the importance of whole wheat and whole wheat based products in preventing major diseases and disease conditions, potentials threats, current lacks, and future prospects.

Keywords: Phytochemicals; Wheat; Polyphenols; Antioxidants; Anti-nutrients

Introduction

Wheat (*Triticum* spp.) is an ancient grain and also one of the leading cereal crops, ranking third, in the world [1]. The centre of origin of wheat is South-western Asia. Hybridization of diploid and tetraploid wheat occurred several thousand years ago resulting in the production of hexaploid wheat (common wheat: *T. aestivum* L.) [2]. Common wheat has been consumed as a food for more than 12,000 years. It was believed that Greek, Roman, Sumerian, and Finnish mythology had god and goddesses of wheat. In the United States, wheat was introduced in the early colonial years. However, it was not until 19th century that wheat cultivation flourished, which was brought by the Russian immigrants who settled in the Kansas [2]. At present, the largest commercial producers of wheat include China, India, United States, Russian Federation, and France [1].

The unique viscoelastic properties of wheat, which allow the formation of a number of products, make it leading choice on our diet plate [3]. The ability of wheat to be well suited for a wide range of agro-climatic zones make it the most cultivated crop throughout the world and in the United States. These two reasons make wheat the largest produced and consumed cereals throughout the world. In the U.S., wheat is grown as winter and spring wheat: winter wheat accounts for 70-80% of total production in the U.S. [4]. The United States Department of Agriculture (USDA) classified wheat as hard red winter wheat, hard red spring wheat, soft red winter wheat, white wheat, and Durum wheat, the use of which may be different depending upon the final product. The composition of wheat varies among genotypes, environment and their interactions, classes of wheat [5], and parts of a wheat grain [6]. A wheat grain is divided into three main parts, the bran, endosperm, and germ. The bran, the outer layer, is composed of fibres (50%), antioxidants, B vitamins, and 50-80% of minerals are composed of iron, copper, zinc and magnesium [7]. Wheat is also a rich source of protein, carbohydrates and fibres as mentioned in several previous studies [3]. The protein content in the germ and aleurone layer is 30%, whereas the pericarp layer contains 10% of the total protein [6]. Though protein contents in wheat aleurone and germ layer are high, the contribution of endosperm to total kernel protein content is relatively higher; about 74% [6].

The health promoting effects of whole wheat or whole wheat based products are mainly due to the presence of phytochemicals in wheat, including phenolic, carotenoids, lignans and vitamin E [8]. Each of these phytochemicals in combination or alone accelerates health-promoting events. Several past studies have shown the effectiveness of polyphenols against oxidative stress and dietary fibre against cancer [9]. The amount of these phytochemicals varies depending upon the fraction of wheat grain and the processing conditions. In a study, environment influences on the total phytochemical concentrations were pronounced more significant than production system [9]. In the recent years, several studies on health benefits of whole wheat are increasing the perception of people and inclination towards whole wheat/whole wheat based products. Due to the association of whole wheat in preventing several diseases, numerous studies have been carried out to date with no sign of decreasing. In future, oxidative stress-related diseases, obesity, cancer, and diabetes are likely to cause large number of deaths. Though several studies have been conducted and much is known about benefits of whole wheat, there remains a lack of information about the phytochemicals present in whole wheat to their possible association with different diseases. This paper reviews phytochemicals present in whole wheat and studies of several health benefits and the health promoting action of whole wheat with special emphasis on oxidative stress, heart diseases, cancer, inflammation, obesity, and immunology carried out in the last decade.

Composition of Whole Wheat

Wheat grain is comprised mainly of starch, proteins, and cell wall polysaccharides (dietary fibre) [5]. These components in combination

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account for 90% of the grain composition on dry weight basis. The minor components present in wheat are lipids, terpenoids, phenolics, minerals, and vitamins. The fat soluble nutrients that include vitamin E (tocopherols and tocotrienols, collectively called as tocols) and the carotenoids are located in wheat flour. In the wheat kernel, tocols are unevenly distributed as the activity of methyltransferases is crucial in determining the relative proportion of vitamins in grains [6]. Tocols scavenge peroxyl radicals, singlet oxygen and nitrogen oxide, which are responsible for the oxidative stress-mediated diseases, such as cancer and coronary heart diseases. However, the bran matrix formed in whole wheat flour may decrease the bioavailability of tocols [8]. According to the U.S. Department of Agriculture's National Nutrient Database, whole-grain wheat flour has 0.71-0.85 mg/g of vitamin E (α-tocopherol). Processing of the whole wheat causes loss of tocols, as they are subjected to oxidation in the presence of heat, light or alkali. During extrusion, around 85% losses of tocols have been reported [9]. Carotenoids are not active as alone but contribute to antioxidant property of wheat [10]. Lutein, β-cryptoxanthin, zeaxanthin (xanthophyll: hydroxylated carbons) and β-carotene (hydrocarbon) is the carotenoids present in wheat [9]. Though wheat flour is not a rich source of carotenoids, it is still an important nutrient considering the amount of whole wheat consumed.

The phytosterols are mainly located in the wheat kernel [9]. The total phytosterols in winter wheat grain range from 0.62-0.96 mg/g dry weight basis [7]. The predominant phytosterol in wheat is the desmethyl sterols (no methyl group at C-4). Campesterol, sitosterol, stigmasterol, avenasterols (desmethyl sterols) and the stanols (saturated sterols) are different phytosterols that have been reported in wheat grains. Phytosterols have been shown to reduce low-density lipoprotein cholesterols and may have a promising effect against cancer [8].

Phenolics, compounds containing a phenol ring and at least one hydroxyl substituent, are the main constituents of wheat. Phenolic compounds are located mainly in the outer barn layers of kernels [7]. Wheat phenolics include the phenolic acids, flavonoids, and alkylresorcinols. Ferulic acid (main phenolic acid in wheat), caffeic acid, sinapic acid, protocatechuic acid, vanillic acid, p-hydroxybenzoic acids, p-coumaric acid, and syringic acid are some phenolic acids present in wheat bran and whole wheat flour [9]. Phenolic acids are mainly known for their antioxidative properties. However, the antioxidative properties of phenolic acids have been reported to decrease when they bind to the protein. The flavonoids are mainly located in the wheat germ (0.09 μmol/g of grain) [11]. Flavonoids have both anti-oxidative and anti-inflammatory activities [12]. Lastly, lignans are the phytoestrogens, which are found in the wheat bran. Though studies remain limited on wheat lignans, their association has been reported against cancer [13].

Oxidative Stress

High reactive molecules, known as free radicals, are produced in our body that causes oxidative stress (imbalance between oxidants and antioxidants) due to the hyper oxidation nature of oxygen [14]. When our body can't cope with the oxidative stress, oxidative damage occurs in the form of many diseases, including but not limited to Coronary Heart Diseases (CHD), cancer and diabetes [14]. However, the regular intake of whole wheat in our diet has been associated with the reduced risk of chronic diseases and oxidative stress related disorders [11]. Several mechanisms that include termination of free radical-mediated oxidative reactions, stimulation of antioxidant enzymes, reduction of peroxides, and chelation of transition metals, are exhibited by dietary antioxidants/polyphenols against the oxidative stress [15]. The beneficial effects of whole wheat are associated with the presence of phenolic, carotenoids, vitamin E, lignans, polysterols and dietary fibre.

Ferulic acid, the main phenolic acids in wheat, has high anti-oxidative activity [16]. For example, several studies have shown its protective properties against cancer, skin diseases, and diabetes [7]. The pericarp, testa, and aleurone layer of wheat contain the highest concentration of polyphenols. Several studies have been conducted to date to show that the polyphenols in wheat and their ability to reduce oxidative stress-mediated disease [17]. In the current years, the focus has been made on identifying the specific effect of wheat components (different polyphenols) in reducing the risk of diseases.

Cancer

Cancer is the second leading disease causing the death of people in the United States after heart disease (Figure 1) [18]. The expression of different types of cancers partially reflects different environmental hazards and lifestyles. For instance, mouth, oesophagus, and stomach cancer are associated with environments lacking good hygiene, foods and food preservation systems, whereas colorectal cancer and breast cancers are closely associated with westernized cultures [19]. Whole wheat is a rich source of dietary fibres that include a non-starch polysaccharide, resistant starches, sugar alcohols, and oligosaccharides. It has been shown that oligosaccharide alone or in combination with probiotics significantly reduced colonic aberrant crypt foci and tumors induced azoxymethane [20]. Resistant starches shown in the whole wheat improved a number of bowel health markers, including increased butyrate [21]. These resistant starches escape digestion and undergo fermentation in the colon to provide a significant amount of short chain fatty acids including butyrate. Butyrate exerts its antineoplastic effect by modulation of gene expression, inhibition of cell growth, increased histone acetylation, and induced apoptosis [22]. Other fermentative activities and short chain fatty acids improve the availability and uptake of cations, such as calcium and magnesium by their opening influence on tight junctions in the colonic epithelium [23]. Lack of fermentation in the large bowel by the usage of antibiotics diminish butyrate and the production of the B vitamins, which otherwise are beneficial in cancer prevention [23].

Phytosterols, phenolics, and selenium shown in whole wheat have anticancer effects [24]. Some compounds act as antioxidants, locking up minerals and trace element capable of producing free radicals from fats [24]. Phenolics present in the whole wheat can act as antibiotics or antioxidants. The presence of the orthophenolics in wheat was correlated significantly with tumour inhibition [25]. The metabolism of lignans (di-phenolics found in whole wheat) has been also been correlated with a reduced risk of breast cancer [26]. Dimethyl benzoquinone (found in wheat germ) was shown to be effective as an anticancer agent in the rodent colon cancer model at low concentrations [27]. Selenium, present in the aleurone layers and germ of wheat, (1-2 ppm) was effective in cancer prevention using the rodent colon cancer model [28]; however, no beneficial effects occurred in older individuals when they were supplemented with dietary Se [29]. An anticancer bio peptide, Lunasin (249.2 μg luanisn/g seed) in wheat was reported [30]. Anti-carcinogenic properties of lunasin were previously reported in soybean [31]. The anti-carcinogenic activity of Lunasin was attributed to its ability to bind to deacetylated histones in the nucleus. According to the study, Lunasin selectively killed the cells that disrupted histone acetylation-deacetylation balance, when oncoprotein inactivates Rb (tumor suppressor). Inactivation of Rb dissociated with the Rb-histone deacetylase complex and this exposure provided a site for lunasin bonding. This binding caused cell death of the transformed cells

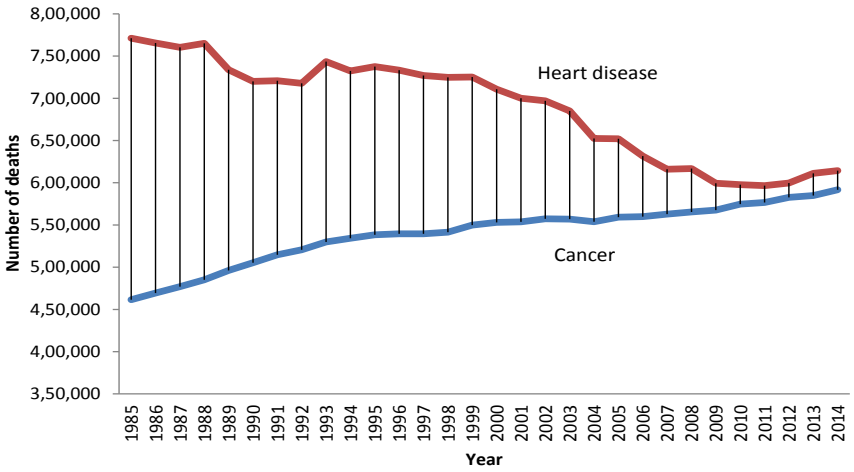

Figure 1: Number of deaths due to heart disease and cancer in the United States from 1985 to 2014. Source: CDC: NCHS, National vital statistics system, mortality.

(cells that were being transferred by disrupting histone acetylation-deacetylation) only.

Wheat extracts also inhibited human colon cancer *in vitro* [15]. The percentage inhibitions of cancer cells were correlated with percentage composition of soluble ferulic acid and p-coumaric acid. Immature wheat kernels were more effective against two colon cancer cell lines (HT-29 and Caco-2) and HeLa cervical cancer cells [32]. Wheat germ extract (Avemar) has been reported to be involved in the apoptosis of MCF-7 breast cancer, Jukrat acute lymphoid leukemia T cell, HL-60 promyelocytic leukemia, H9 human lymphoid cell, and gastric cancer cells [33,34]. Avemar also was able to reduce nucleic acid synthesis in the tumor cell by altering the glucose uptake of cancer cells [33].

Whole grains also contain a significant amount of anti-nutrients (not all anti-nutrients are harmful to health), one such is a protease inhibitor. Protease inhibitors were shown to have inhibitory actions against cancer by the suppression of expression of neoplasia in cells (cells are already exposed to cancer) and by inhibiting the tumour promotion [22].

Inflammation

Inflammation is the outcome of the innate immune system triggered by obnoxious stimuli, microbial pathogen, and injury [35]. Continuous activation of the immune cells gives rises to the chronic inflammation. The initiation and progression of the cardio vascular disease, arthritis, diabetes, pulmonary disease, autoimmune diseases etc. accelerate chronic inflammation [36]. Chronic inflammation elevated levels of pro-inflammatory cytokines and acute phase proteins, such as interferons (IFNs), interleukin (Il)-1, Il-6, tumor necrosis factor-α (TNF-α), and C-reactive protein (CRP) [36]. Ferulic acid has a high anti-oxidative activity and is also actively involved in the production of insoluble dietary fibre. The inhibitory activities of the ferulic acid against carcinogenic diseases has been attributed to the prevention of the formation of carcinogen compound from precursor compound and the blockage of the reaction of carcinogen with cellular macromolecules [22]. As stated previously, ferulic acid is present in relatively high quantities in wheat, indicated that wheat or its co-products could cause this mechanism. However, more studies are obviously needed to support this hypothesis

Other phytochemicals present in the whole grain play their own role to suppress carcinogenic or cardiovascular diseases, which leads to the chronic inflammation.

Cardiovascular Diseases

Lipid deposition, oxidative stress, vascular inflammation, smooth muscle cell differentiation, and endothelial dysfunction play a vital role in the formation and progression of the atherosclerotic plaque [36]. Nutrition interventions in the food habits have been associated with the lower risk of coronary heart diseases (CHD) [37]. Serum low-density lipoprotein (LDL) cholesterol (major risk factor for CHD) was reported to decrease with increased intake of soluble fibre, plant sterols and higher intake of whole wheat [38]. In another study, it was shown that phytoestrogen in isolation, but is also present in wheat, binds to a specific intracellular receptor, which in turn protects blood vessels from atherosclerosis [39]. This hormone promotes appropriate vasodilation of coronary arteries when increased blood supply is required. In yet another study, the phytoestrogen from whole wheat (enterolactone) was shown to have a protective effect on vascular events related to arterial spasm [40]. The consumption of 48-50 g of whole wheat (3-5 serving/d) was able to reduce the risk of CVD by 21% [41]. The total and LDL-cholesterol concentration were also significantly lower in their study.

Increased LDL cholesterol levels, hypertension, diabetes, obesity, sedentary life style, low antioxidant vitamin status, and hyperhomocysteniemia are some of the major risk factors for CHD [42]. Several studies on whole wheat have demonstrated the potential of whole wheat to alter these risk factors [40]. Whole wheat phytochemicals, beta-sitosterol (lowers cholesterol), dietary fibres, resistant starch, anti-nutrients, such as phytic acids, and tannin have been shown to lower plasma cholesterol and triglycerides [43].

Immunology

The immune system is a complex web of cellular interactions, which is influenced by health, genetics, and consumption of the foods [44]. Whole wheat contains a plethora of bioactive components, the consumption of which influences biochemical reactions in our body. Ferulic acid, the major bioactive component in whole wheat, contributes the functional immunity mostly by improving gamma delta T (γδ T) cell proliferation and function in the body [29]. γδ T cells are important immune cells that have the properties of innate and acquired immune systems [45]. Ferulic acid acted as a pathogen-

associated molecular pattern (PAMP) for the γδ T cells [29]. The γδ T cells lysed cells via perforin of Fas-ligand-dependent pathways. These cells also secreted cytokines and chemokines in order to recruit monocytes and neutrophils to the site of inflammation. γδ T cells either recognize PAMPs, phosphor antigens or non-peptide, lipid antigens, and respond directly to Toll-like receptor (TLR) ligands without the presence of antigen presenting cells. In a recent study in mice, ferulic acid modulated the function of dendritic cells (antigen presenting cells) to promote interferon (IFN)-gamma production by activated T cells. Further it was explained that ferulic acids affected the Th2 based immune response. In addition, ferulic acid was determined to act as an antiallergic in treating Th2 mediated allergenic response [46].

Studies using dietary fibres present in whole wheat were able to stimulate the immune function via production of short-chain fatty acids (SCFAs) [47]. The addition of SCFAs to parenteral feeding has shown to increase T helper cells, macrophages, neutrophils, and cytotoxic activity of natural killer cells in animal studies [48]. Celiac disease (caused due to dietary intolerance of wheat), a chronic inflammation of the bowel, results from an autoimmune response due to the binding of gluten peptides to T cells of the immune system (only to those people who are allergic to wheat) with the human leucocyte antigens (HLA) DQ2 and DQ8 [7]. These bound peptides are recognized by specific CD^{4+} T cells and then releases inflammatory cytokinesis, which flattens the intestinal epithelium [7].

Gastrointestinal Microbiota

Arabinoxylan and cellulose (important components in whole wheat) are poorly fermented in the gut [49] and have high potential to improve/maintain a healthy gut. Consumption of whole grain was shown to increase bifidogenic effect: some strains of Bifidobacterium are markers of healthy gut [15]. An increase in butyrate-producing bacteria, Roseburia, Eubacterium rectale and the *Clostridium leptum*, contributed to host colonic epithelial cell energy during the fermentation of carbohydrates from the whole wheat [50]. A study has shown that dietary fibers provided significant health benefits by increasing viscosity, which delays gastric emptying and limits glucose diffusion towards the enterocytes for absorption [15]. Dietary fibre intake has also been associated with an increase in satiety thus help to control body weight [51]. The mechanism for this may be due to the hormonal effects mediated by the reduction of insulin secretion, the metabolic effect mediated by increased fat oxidation and colonic effects via SCFA production [22]. Decreases in the diversity of the microbiota are associated with increased risk of obesity and disease [43]. Whole wheat consumption increased the microbiota. Another compound, lignan in whole wheat was able to protect against hormonally mediated diseases [22]. Plants lignans are converted by gut bacteria to the mammalian lignans enterolactone and enterodiol. Saturated fat exhibits an antimicrobial effect and consequently reduced diversity [49]. Considering that whole wheat is lower in the total fat content but high in high unsaturated fat, these fats have been shown to produce a healthy microbial effect [48]. An increase in the Bifidobacteria and decrease in Coriobacteriaceae resulted when whole wheat was incorporated in diet. Bifidobacteria was correlated with plasma HDL-concentration, while Coriobacteriaceae was correlated with non-HDL cholesterol [48].

Metabolic Syndrome

Metabolic syndrome is a pattern of metabolic disturbances associated with increased risk of type 2 diabetes, raised blood pressure, dyslipidaemia, and obesity [52]. Several studies on intake of whole grains have been reported that consumption of wheat was negatively associated with metabolic syndrome [35,53]. Whole wheat flour has been able to improve glycaemic control and insulin sensitivity, decrease blood pressure and produce a healthy body mass index (BMI) as discussed in reference [22]. The mechanism for the above response was due to the presence of bran, which decreased glucose absorption and produced SCFA from the fermentation of resistant carbohydrates, which in turn improved insulin sensitivity [53].

Yet another study, tocotrienols in whole wheat decreased the risk of heart disease whereas β-sito sterol was associated with a decrease in cholesterol [34]. A study with ferulic acid, which is the major component in whole wheat, showed that ferulic acid converted into DHFA by microbiota in gut resulting an increase in Bacteroidetes and Firmicutes [54]. γ–oryzanol (γ–oryzanol content in wheat bran ranged from 300-390 mg/kg) observed in wheat barn showed to lower serum cholesterol effectively than tocopherols and tocotrienols [55]. Low-density cholesterol (LDL) was shown to be reduced by 12% and ratio of LDL to HDL (high-density lipoprotein) cholesterol was decreased by 19% when diets containing γ–oryzanol were fed to rats (Mitchell et al., 1996). That arabinoxylan reduced the postprandial glucose level due to its high viscosity [56]. As a result, arabinoxylan reduced small intestinal motility resulting in delayed glucose absorption; hence a flat postprandial glucose response was observed.

Whole Wheat Prospects and Lacks

The diversity of the nutraceuticals present in whole wheat makes this bran an excellent commodity on our diet plates. Several studies have been carried out over a decade, to identify its multiple health benefits including cellular oxidation mediated diseases, cancer, atherosclerosis, inflammation, obesity, and diabetes [5,22] as described throughout this documents. The presence of polyphenols in whole wheat was correlated with the reduced risk of the diseases. However, most of the studies were focused either on the individual action of the polyphenols, supplementation of the wheat/wheat extract *in vitro*. Only very few studies reported the effect on health due to the overabundance of specific phytochemicals. Will the effect be beneficial or detrimental? Also, the bioavailability of the proposed phytochemicals in presence of anti-nutritional factors is another crucial concern. Several studies have reported the binding of polyphenols with trypsin inhibitors during digestion [57]. The fact that humans may have other diseases and allergic response, the beneficial effects of polyphenols on these groups of people is not yet known. Will the unhealthy people have a similar effect or a new health complication may take place? Moreover, the analytical method used can vary the amount of polyphenols [58]. A unique and consistent approach to quantify compounds may give a reliable results considering their chemical diversity and their interactions with a given food matrix. Besides above-mentioned questions, a number of phytochemicals in wheat are largely depended on the growing conditions of wheat and therefore, probably each food type will have to be optimized on its own merit.

Given the fact that polyphenols are produced in plants to protect them against stresses (biotic and abiotic), how relevant it is in terms of total production of wheat. There lacks the specification in the classes of wheat that were being used in the study. Compared to white wheat, red wheat contains higher levels of polyphenols, but what about the bioavailability of those compounds. An abundance of polyphenols does not really assure higher bioavailability. Studies on diverse group of the population also may help to pronounce effect of phytochemicals on wheat. In presence of stresses, the total production decreases, but polyphenols may increase in those grains. Will the phytochemicals derived from unhealthy grains be useful to us?

In the recent years, besides health benefits from whole wheat several studies have shown the negative or possible problems due to the consumption of whole grains. Given the possibility of contamination of heavy metals on whole wheat and their possible adverse effects on human health, it is important to study if whole wheat is actually beneficial or not. Also, the contamination of whole wheat by nanoparticles such as zinc oxide and titanium dioxide [59,60] and their potential effects to health has been a major concern regarding the use of whole wheat in our diet. The ability of nanoparticles to cross the blood-brain barrier, move to other vital organs, and possible damage of DNA [61] makes whole wheat questionable in its potential benefits. The contamination of nanoparticles from the different sources in an environment to plant and finally to grains [59] is the serious issue and more studies need to be carried out. The amount of heavy metals that reaches on our diet plate, their potential to exacerbate other benefits either by binding with phenols or by acting as inhibitory agent: these all areas need to be addressed.

Concluding Remarks

Although presence of anti-nutrients and binding of phenolic acids with protein may have adverse effect on health, whole wheat and whole wheat based products played a great role to improve health by preventing major diseases and disease conditions. However, thorough study on the types of wheat, interaction of the phytochemicals with other proteins, heavy metal accumulation in wheat bran, bioavailability of compounds in different wheat products need to be addressed in future, which will give us a big picture of the beneficial effects of one of the largely consumed products in world.

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Phenolic Content and Antioxidant Capacity of Selected Cucurbit Fruits Extracted with Different Solvents

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Abstract

Cucurbits are major economically important species of plants; particularly those with edible fruits having nutritional significance. The present work was to investigate the polyphenolic content and antioxidant capacity of peels and pulps of four cucurbit fruits, namely pumpkin, ash gourd, watermelon and muskmelon. The solvent systems used were methanol, ethanol and acetone at three different concentrations in distilled water (50, 70, and 100%) and 100% distilled water. The extracts were analyzed for their total phenolic content, total flavonoid content and antioxidant activities using Ferric Reducing Antioxidant Power assay (FRAP assay), DPPH free radical-scavenging assay and ABTS radical scavenging capacity. The result showed that the highest extraction was by 50% acetone in case of peels and 50% ethanol in case of pulp. The best solvent was 50% acetone as it gave highest yield as well as showed highest correlation between various assays. The polyphenolic content and the antioxidant activity were high in peels than pulps. The muskmelon fruit extracts (peel and pulp) showed highest antioxidant activity. High polyphenolic content showed significant correlation with high antioxidant activity. The result indicated that these cucurbit fruit are good source of natural antioxidants which can be utilized as an ingredient to functional foods.

Keywords: Cucurbits; Antioxidant activity; Polyphenolic content; Solvent extraction

Introduction

Free radicals, Reactive Oxygen Species (ROS), and Reactive Nitrogen Species (RNS) are implicated in numerous pathological conditions such as inflammation, metabolic disorders, cellular aging, reperfusion damage, atherosclerosis, and carcinogenesis [1,2]. The high levels of ROS and free radicals cause damage to nucleic acids, proteins, and membrane lipids. The antioxidants in diet would terminate attacks by the free radicals and reduce the risks of these diseases [3]. Many plants contain antioxidants viz. vitamin C, vitamin E, carotenoids, polyphenols, phenolic acids, phenolic diterpenes, flavonoids, catechins, procyanidins and anthocyanins. The focus has been shifted to naturally occurring antioxidant. The use of natural antioxidant are considered to be safe rather than synthetic as latter may show carcinogenic potential [4]. Various plant materials with great antioxidant potentials have been identified and are widely used in food, pharmaceuticals, cosmetics and diverse fields related to the utilization of antioxidants. Some plants are commonly cultivated for their culinary purposes but their potential as source of antioxidants is less exploited. Thus, it provides a new approach to develop new sources of antioxidants that can be used in food, neutraceutical and other fields [5].

The proper recovery of phenolic compounds is one of the important tasks. Solvent extraction is frequently used method for the extraction of these compounds from plant material. Different types of solvents are used for the preparation of extracts from the plant materials since solvent system is easy to use and efficient for extraction of different compounds. The physical and chemical property of the sample, type of solvent, extraction time and temperature and sample to solvent ratio effect the extraction yield of the compounds [6]. Solvent polarity and the solubility of the phenolics in the solvent are dependent on each other which affect the recovery of the polyphenols from the plant materials [7]. Polyphenols are mostly extracted from plant matrix by using polar solvents. Basically, the aqueous mixture containing acetone, ethanol, methanol and ethyl acetate are the most suitable solvent for the recovery of polyphenolic compounds [6].

Cucurbits play an important role in human consumption and has higher consumption rate in tropical regions [8]. The fruits are good source of vitamins, minerals and also hold good antioxidant and nutraceutical potential. Watermelon exemplifies one of the most widely cultivated crops in the world, occupying the largest production of all Cucurbits. The watermelon fruit possess high antioxidant potential and free radical scavenging activity in all parts namely peel, pulp and seed [9]. Pumpkin is cultivated worldwide for its nutritional and medicinal importance. Each pumpkin part contains a significant amount of antioxidants, tocopherols, and carotenoids [10]. Muskmelon pulp extracts have shown high antioxidant potential and anti inflammatory activity [11]. Ash gourd is usually renowned for its antioxidant and medicinal property mainly in Asian countries [12].

The literature related to the antioxidant potential of peel and pulp of cucurbit fruits as well as effect of different solvent system on extraction of phenolic compounds in these fruits was scarce. The objective of this study was to determine the effectiveness of different solvent systems i.e., methanol, ethanol, acetone and aqueous mixture of these in different proportions for extraction of polyphenolic compounds from peel and pulp of cucurbits and to investigate the antioxidant potential of the extracts of these fruits in different solvents.

Materials and Methods

Plant materials

Fresh fruit samples were collected at different times from local markets in Allahabad region of Uttar Pradesh, India. Samples included

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pumpkin (*Cucurbita maxima*), ash gourd (*Benincasa hispida*), watermelon (*Citrullus lanatus*) and muskmelon (*Cucumis melo*). All the fruits were free from any physical and microbial damage. Each fruit was identically selected considering the quality traits in terms of shape, size, color, and ripening stage.

Chemicals and reagents

2,4,6-Tri-(2-pyridyl)-s-triazine (TPTZ), 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox), Folin–Ciocalteu reagent (FCR), Quercetin were purchased from Sigma- Aldrich Co. (St. Louis USA). Gallic acid (purity >99.0%), trichloroacetic acid (TCA), ferric chloride, sodium carbonate, sodium acetate trihydrate, sodium nitrite, sodium hydroxide, aluminium chloride, acetone, methanol, ethanol were obtained from Merck (Germany). The other chemicals were of analytical grade and the water used was deionized. These chemicals were used as such without undergoing further purification.

Extraction

Fruits were washed with distilled water and separated into different parts i.e., peel and pulp. After that, fruit parts (2.5 g each) were cut into small pieces and blended for 3 min. Then the sample were placed in capped centrifuge tubes and extracted with 10 ml of organic solvent on an orbital shaker (Remi IS 24BL) set at 200 rpm for 2 hrs at room temperature (25 ± 2°C). The samples were again centrifuged using tabletop centrifuge (Remi) for 10 min at 1000 rpm. Next, the samples were filtered through Whatman filter paper No. 1 and the process was repeated twice with each residue obtained after filtration. The filtrate of each extraction was collected simultaneously. The extracts were brought down to dryness using a rotary vacuum evaporator (IKA, RV10) at 50°C. Finally the dried extracts were stored at 4°C to prevent the degradation of compounds. The extraction process was carried out in triplicate, using different fruit samples each time. The solvent system used was methanol, ethanol and acetone at three different concentrations in distilled water (50, 70, and 100%) and 100% distilled water (H_2O).

Total phenolic content (TPC)

The total phenolic content of the samples was determined spectrophotometrically according to the Folin–Ciocalteau method [13]. 0.1 mL of each extract was diluted with deionised water to 4.8 ml, and 0.3 ml Folin - Ciocalteau reagent was added and shaken. After 8 min, 0.9 ml of 20% sodium carbonate was added along with mixing. The solution was incubated at 40°C for 30 min before recording the absorbance at 765 nm in spectrophotometer (Model Evolution 600, Thermoscientific, US). The measurement was compared to a standard curve of Gallic acid solutions (20, 40, 60, 80, 100 mg/L) and results were expressed on fresh weight basis as milligrams of gallic acid equivalents per 100 g (GAE/100 g) samples for the extracts.

Total Flavonoid content (TFC)

The total flavonoid content was measured using aluminum chloride colorimetric assay by Zhishen et al. [14] with slight modification. 1 ml of sample extracts was added to flask containing 4 ml of water. To the above mixture, 0.3 ml of 5% $NaNO_2$ was added. After 5 min, 0.3 ml of 10% $AlCl_3$ and after 6 min, 2 ml of 1 M NaOH was added. The total volume was made up to 10 ml with distilled water. Then the solution was mixed well and the absorbance was measured against a freshly prepared reagent blank at 510 nm. A calibration curve was prepared using a standard solution of quercetin (20, 40, 60, 80 and 100 mg/L). The results were expressed on a fresh weight basis as mg quercetin equivalent (QE)/100 g of sample.

Ferric-reducing antioxidant power (FRAP) assay

The FRAP assay was carried out according to the method described by Benzie and Strain [15], Benzie and Szeto [16] with slight modification. Briefly, the FRAP reagent was prepared from sodium acetate buffer (300 mM, pH 3.6), 10 mM TPTZ solution (40 mM HCl as solvent) and 20 mM iron(III) chloride solution in a volume ratio of 10:1:1, respectively. One hundred microlitres of the diluted sample was added to 3 mL of the FRAP reagent. The absorbance of the mixture was measured at 593 nm after 30 min incubation. The standard curve was prepared using $FeSO_4.7H_2O$ solution (200, 400, 600, 800, 1000 µM), and the results were expressed on fresh weight basis as µM of ferrous equivalent Fe (II) per g of sample.

DPPH radical scavenging activity

The DPPH radical scavenging activity of the extracts were evaluated by 1,1-diphenyl 2-picryl-hydrazil (DPPH) using the method given by Bhat and Karim [17]. An aliquot (100 µL) of fruit extract was mixed with 3.9 ml of 0.1 mM DPPH methanolic solution. The mixture was vortexed thoroughly and kept in the dark for 30 min. The absorbance was measured at 515 nm, against a blank of methanol. The radical scavanging activity was calculated using the ratio: (Acontrol – Asample/Acontrol) × 100, where Acontrol is the absorption of the DPPH solution and A sample is the absorption of the DPPH solution after the addition of the sample. Results were expressed as percentage of inhibition of the DPPH radical.

Antioxidant capacity determined by radical cation ($ABTS^{\cdot+}$)

ABTS assay was carried out according to the method described by Re et al. [18]. ABTS radical cation ($ABTS^{\cdot+}$) stock solution was produced by reacting 7 mM ABTS solution with 2.45 mM potassium persulphate in volume ratio of 1:1 and allowing the mixture to stand in the dark at room temperature for 12–16 hrs before use. The $ABTS^{\cdot+}$ solution was diluted with solvent to an absorbance of 0.70 ± 0.02 at 734 nm. After addition of 100 µL of sample or trolox standard to 2 mL of diluted $ABTS^{\cdot+}$ solution, absorbance at 734 nm was measured at exactly 6 min. The calibration curve between absorbance and known solutions of Trolox (200, 400, 600, 800, 1000 µM) was then established. Results were expressed as Trolox equivalent antioxidant capacity (TEAC µmol Trolox/g).

Statistical analysis

All the analysis was carried out in triplicate and values were expressed as means ± standard deviations. Data were analyzed using SPSS version 20 for Window software (IBM corp.) Analysis of variance (ANOVA) and Duncan's multiple range method were used to compare any significant differences between solvents and samples. Differences were considered significant at $P<0.05$. Correlation analyses between antioxidant activities and polyphenolic content were performed using Pearson's correlation coefficient (r).

Results and Discussion

Extraction yield

Extraction is an important initial step for the recovery and isolation of bioactive compounds from plant samples. The efficiency of extraction as well as yield depends on type of solvents used, solubility

and polarity of the compounds with the solvent, time and temperature of extraction [19]. Sulaiman et al. [20] soaked the sample paste in solvent for 1 hrs and centrifuged it. Musa et al. [21] used 300 rpm for 1 hrs for extraction process which gave better results than maceration. Therefore, the samples were subjected to high speed shaking (200 rpm) for 2 hrs as it have an effect on the morphology of the sample matrix which causes the bioactive compounds to get released more quickly and also increases the extraction process. The extraction yield of peels and pulps in different solvents are shown in Table 1. The extraction yield of the extracts in different solvents ranges from 2.85 ± 0.19 to 10.18 ± 0.66% in a decreasing order of 50% acetone>50% ethanol>50% methanol>70% acetone>70% ethanol>70% methanol>100% etone>100% methanol>100% ethanol>water.

The result showed that the extraction yield of pure acetone was higher than that of pure methanol and pure ethanol. This increase in yield may be due to aprotic nature of acetone as compared to other solvents. It was also found that the extraction yield of the extract with water was somewhat less than that of the extracts of pure solvents. The extraction yield showed increment as the concentration of water in the solvents was increased. This may be due to the extraction of chemical compounds which are soluble in organic solvents and/or water. The phytochemical analysis of the fruits showed the presence of various compounds like tannins, glycosides, terpenoides, carotenoids, phytosterols etc. Cucurbitacins is the most common terpenoides [22]. Tannins are soluble in water or organic solvents like alcohol, acetone while glycosides are soluble in water and insoluble in organic solvents. Terpenoids, carotenoids and phytosterols are soluble in organic solvents and insoluble in water. Therefore the aqueous mixtures of organic solvents gave higher extraction yield than pure solvents and water. Similar results were shown in medicinal plants [23] and rice bran [24].

Polyphenol content

The phenolic compounds in plants are considered to scavenge free radicals and thus it is opinioned that the antioxidant activities shown by plant materials occur due to presence of phenolic compounds [25]. These compounds have the capability to decrease the concentration of free or singlet oxygen, donate hydrogen atom to free radical, decomposition of free radicals to non radical and to prevent removal of hydrogen by breaking chains. Experimental results are similar to those obtained in the phenolic extraction from other fruits [26,27].

Tables 2 and 3 showed the total phenolic content of pulps and peels respectively in different extracting solvents. The TPC of the pulp was highest for muskmelon pulp followed by watermelon, pumpkin and ash gourd in all solvents while in case of peel, it was highest for muskmelon

Solvents	Pumpkin		Ash gourd		Watermelon		Muskmelon	
	Pulp	Peel	Pulp	Peel	Pulp	Peel	Pulp	Peel
Water 100	3.26 ± 0.28^{a}	4.02 ± 0.17^{a}	2.85 ± 0.19^{a}	3.70 ± 0.15^{a}	3.47 ± 0.22^{a}	4.56 ± 0.2^{a}	3.50 ± 0.34^{a}	5.08 ± 0.12^{a}
Acetone: Water								
100:0	4.44 ± 0.30^{c}	6.27 ± 0.12^{c}	3.68 ± 0.25^{c}	5.26 ± 0.20^{c}	4.71 ± 0.20^{c}	5.41 ± 0.13^{b}	4.96 ± 0.11^{c}	5.97 ± 0.11^{b}
70:30	6.05 ± 0.06^{e}	8.42 ± 0.30^{e}	5.57 ± 0.28^{d}	7.32 ± 0.21^{e}	6.09 ± 0.12^{e}	7.48 ± 0.36^{d}	6.43 ± 0.11^{f}	8.79 ± 0.37^{d}
50:50	8.39 ± 0.14^{g}	10.18 ± 0.66^{f}	7.88 ± 0.08^{f}	9.13 ± 0.10^{f}	8.05 ± 0.09^{g}	9.71 ± 0.39^{e}	8.63 ± 0.27^{i}	10.11 ± 0.11^{f}
Methanol: Water								
100:0	3.67 ± 0.19^{b}	5.66 ± 0.31^{b}	3.46 ± 0.32^{bc}	4.93 ± 0.16^{bc}	4.34 ± 0.13^{b}	4.76 ± 0.31^{a}	4.76 ± 0.35^{bc}	5.23 ± 0.23^{a}
70:30	5.43 ± 0.23^{d}	7.37 ± 0.26^{d}	5.29 ± 0.16^{d}	6.61 ± 0.34^{d}	5.64 ± 0.19^{d}	6.56 ± 0.27^{c}	5.92 ± 0.16^{e}	7.27 ± 0.18^{c}
50:50	7.39 ± 0.16^{f}	9.82 ± 0.19^{f}	7.04 ± 0.06^{e}	9.04 ± 0.10^{f}	7.47 ± 0.27^{f}	9.14 ± 0.18^{e}	7.45 ± 0.29^{g}	9.56 ± 0.34^{e}
Ethanol: Water								
100:0	3.50 ± 0.20^{ab}	5.46 ± 0.14^{b}	3.30 ± 0.19^{b}	4.80 ± 0.21^{b}	4.19 ± 0.11^{b}	4.61 ± 0.15^{a}	4.43 ± 0.19^{b}	5.16 ± 0.24^{a}
70:30	5.52 ± 0.23^{d}	7.58 ± 0.29^{d}	5.44 ± 0.22^{d}	6.74 ± 0.26^{d}	5.83 ± 0.24^{de}	6.89 ± 0.18^{c}	6.04 ± 0.12^{ef}	7.40 ± 0.25^{c}
50:50	7.44 ± 0.21^{f}	9.87 ± 0.12^{f}	7.34 ± 0.06^{e}	9.07 ± 0.09^{f}	7.63 ± 0.15^{f}	9.52 ± 0.28^{ef}	7.89 ± 0.21^{h}	9.74 ± 0.25^{ef}

All values are means ± standard deviations of data from three independent experiments
Different superscripts (a, b, c, d…..i) in the same column indicate significant difference (P<0.05)

Table 1: Extraction yield of pulp and peel extracts of cucurbit fruits from different solvent.

Solvents	Pumpkin		Ash gourd		Watermelon		Muskmelon	
	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)
Water 100	13.92 ± 1.49^{a}	3.79 ± 0.51^{a}	11.63 ± 1.00^{a}	2.54 ± 0.28^{a}	18.47 ± 0.94^{a}	4.60 ± 0.28^{a}	22.75 ± 0.95^{a}	5.70 ± 0.28^{a}
Acetone: Water								
100:0	21.59 ± 0.97^{c}	5.30 ± 0.45^{b}	19.33 ± 0.96^{c}	4.49 ± 0.41^{b}	24.41 ± 1.15^{bc}	5.45 ± 0.41^{b}	27.76 ± 1.28^{b}	6.26 ± 0.46^{ab}
70:30	24.86 ± 0.91^{de}	6.80 ± 0.46^{c}	22.63 ± 1.33^{de}	5.70 ± 0.37^{c}	28.94 ± 0.93^{de}	7.24 ± 0.43^{cd}	33.47 ± 0.75^{d}	8.39 ± 0.29^{cd}
50:50	32.42 ± 1.08^{fg}	10.48 ± 0.61^{e}	28.80 ± 0.93^{f}	7.92 ± 0.52^{d}	37.71 ± 1.08^{g}	10.57 ± 0.38^{f}	42.27 ± 1.07^{f}	11.84 ± 0.53^{f}
Methanol: Water								
100:0	19.36 ± 0.99^{b}	4.90 ± 0.43^{b}	17.31 ± 0.81^{b}	3.80 ± 0.35^{b}	22.80 ± 0.93^{b}	5.12 ± 0.21^{b}	27.75 ± 1.26^{b}	6.22 ± 0.36^{ab}
70:30	24.38 ± 1.07^{d}	6.90 ± 0.41^{c}	21.05 ± 0.89^{cd}	5.42 ± 0.46^{c}	27.45 ± 0.95^{d}	6.81 ± 0.31^{c}	31.40 ± 0.73^{c}	7.87 ± 0.30^{c}
50:50	30.69 ± 1.04^{f}	9.84 ± 0.44^{e}	27.72 ± 0.93^{f}	7.62 ± 0.49^{d}	34.56 ± 1.08^{f}	9.68 ± 0.42^{e}	38.50 ± 1.08^{e}	10.78 ± 0.35^{e}
Ethanol: Water								
100:0	21.45 ± 0.90^{b}	5.68 ± 0.42^{b}	20.22 ± 0.95^{c}	4.48 ± 0.31^{b}	25.63 ± 1.10^{c}	5.73 ± 0.40^{b}	30.39 ± 1.13^{c}	6.86 ± 0.41^{b}
70:30	24.38 ± 1.06^{c}	7.73 ± 0.40^{d}	23.41 ± 1.28^{e}	5.82 ± 0.39^{c}	30.14 ± 0.85^{e}	7.56 ± 0.31^{d}	34.50 ± 0.99^{d}	8.62 ± 0.40^{d}
50:50	33.48 ± 1.05^{g}	11.72 ± 0.50^{f}	29.11 ± 0.97^{f}	8.00 ± 0.61^{d}	39.94 ± 0.89^{h}	11.14 ± 0.36^{f}	43.75 ± 1.65^{f}	12.32 ± 0.52^{f}

All values are means ± standard deviations of data from three independent experiments
Different superscripts (a, b, c, d…..h) in the same column indicate significant difference (P<0.05)

Table 2: Total Phenolic content (TPC) and Total flavonoid content (TFC) in pulp of four cucurbits using different solvents.

Solvents	Pumpkin		Ash gourd		Watermelon		Muskmelon	
	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)	TPC (mg GAE/100 g)	TFC (mg QCE/100 g)
Water 100	30.22 ± 1.08^{a}	7.29 ± 0.40^{a}	25.47 ± 1.06	5.60 ± 0.35^{a}	30.45 ± 1.09^{a}	5.75 ± 0.26^{a}	44.22 ± 1.00^{a}	9.04 ± 0.67^{a}
Acetone: Water								
100:0	42.47 ± 0.66^{b}	10.93 ± 0.43^{b}	38.95 ± 0.89^{c}	8.63 ± 0.36^{c}	34.53 ± 1.06^{b}	7.76 ± 0.37^{c}	47.53 ± 1.10^{c}	10.41 ± 0.34^{b}
70:30	52.33 ± 0.81^{d}	14.80 ± 0.40^{d}	46.42 ± 0.95^{d}	11.62 ± 0.38^{d}	43.64 ± 1.21^{d}	10.95 ± 0.30^{f}	55.83 ± 0.58^{e}	13.99 ± 0.35^{c}
50:50	63.30 ± 1.05^{f}	20.21 ± 0.65^{f}	53.53 ± 1.09^{f}	15.18 ± 0.53^{f}	48.63 ± 1.10^{e}	13.65 ± 0.53^{h}	67.45 ± 0.95^{g}	18.93 ± 0.47^{e}
Methanol: Water								
100:0	41.66 ± 1.64^{b}	10.98 ± 0.37^{b}	34.67 ± 1.15^{b}	7.78 ± 0.25^{b}	30.59 ± 1.07^{a}	6.76 ± 0.32^{b}	45.92 ± 0.55^{b}	10.45 ± 0.36^{b}
70:30	50.17 ± 1.04^{c}	13.81 ± 0.23^{c}	44.64 ± 1.03^{d}	11.27 ± 0.45^{d}	35.54 ± 1.05^{bc}	8.94 ± 0.54^{d}	54.29 ± 0.89^{d}	13.62 ± 0.22^{c}
50:50	59.38 ± 1.05^{e}	18.67 ± 0.33^{e}	49.81 ± 1.06^{e}	14.26 ± 0.30^{e}	43.78 ± 1.11^{d}	12.38 ± 0.33^{g}	63.74 ± 0.70^{f}	17.91 ± 0.40^{d}
Ethanol: Water								
100:0	43.42 ± 0.95^{b}	10.79 ± 0.56^{b}	35.48 ± 1.15^{b}	8.03 ± 0.46^{bc}	32.24 ± 1.09^{a}	7.25 ± 0.33^{bc}	46.92 ± 0.45^{bc}	10.72 ± 0.3^{b}
70:30	51.41 ± 1.13^{cd}	14.62 ± 0.29^{d}	45.75 ± 1.17^{d}	11.45 ± 0.33^{d}	37.37 ± 1.11^{c}	10.09 ± 0.18^{de}	54.97 ± 0.84^{de}	13.73 ± 0.43^{c}
50:50	60.90 ± 1.9^{e}	19.48 ± 0.75^{f}	50.67 ± 0.92^{e}	14.35 ± 0.38^{e}	45.35 ± 1.00^{d}	13.07 ± 0.20^{h}	64.67 ± 0.78^{f}	18.16 ± 0.33^{d}

All values are means ± standard deviations of data from three independent experiments
Different superscripts (a, b, c, d…..h) in the same column indicate significant difference (P<0.05)

Table 3: Total Phenolic content (TPC) and Total flavonoid content (TFC) in peel of four cucurbits using different solvents.

followed by pumpkin, ash gourd and watermelon. These result showed that muskmelon fruit have higher polyphenolic content than the other cucurbits. Most of the extracts differed significantly (P<0.05) in their total phenolic content. Furthermore, it was found that 50% aqueous ethanol and 50% aqueous acetone showed no significant difference except in case of watermelon and both occurred as most effective solvent for the extraction of TPC from pulp of each fruit while in case of peels, 50% aqueous acetone was the most effective solvent. The least effective solvent was water as it may be due to insolubility of some complex phenolic compounds in water.

Flavonoids are widely distributed group of phenols which act as effective antioxidants [28]. Table 2 showed that in case of pulp, the TFC content was highest in muskmelon and lowest in ash gourd while the watermelon pulp showed higher in TFC content than pumpkin pulp in some selected solvents (50% acetone, 70% acetone, 100% acetone, 100% methanol, 100% ethanol and water) and in other solvents pumpkin pulp showed higher value than watermelon. In case of peels, except in water extract the highest TFC content was in pumpkin peel followed by muskmelon, watermelon and ash gourd respectively as shown in Table 3. The TFC content showed the similar trend as TPC i.e., for pulp, 50% aqueous ethanol and 50% aqueous acetone showed no significant difference except in case of pumpkin while in case of peel, it was 50% aqueous acetone showed highest values. The results indicated that solvent polarity also effect the flavonoid content extraction.

Effect of solvent system

The solubility of chemical compounds of any sample is influenced by the difference in polarities of the solvents used for extraction. Therefore, it is very important to select an appropriate solvent for determination of TPC, TFC and other antioxidant compounds present in a sample [29]. Extraction of phenolic compounds from plant materials by using different solvents, such as acetone, methanol, ethanol and their aqueous mixture have been reported by various authors [30,31]. From the results shown in Tables 2 and 3, it is evident that the recovery of phenolic compounds was dependent on the type of solvent used and its polarity. Among all the extracts, 50% acetone was found to be the most efficient solvent for extracting phenolic compounds in case of peels whereas 50% ethanol in case of pulps. The recovery of total phenolic compounds was least in pure distilled water. These results may suggest the use of 50% acetone and 50% ethanol for extraction of phenolic compounds in cucurbits. Previous studies showed that the mixture of ethanol and water are usually used for the extraction of phenols from plant materials as it can dissolve wide range of phenolic compounds [17,32,33]. Other than ethanol-water mixture, acetone-water mixture can be used for the higher extraction of polyphenolic compounds from plant materials [20,21,34].

Antioxidant capacity

Fruits and vegetable contain variety of compounds showing antioxidant properties. Different methods have been developed to determine the antioxidant activities of different plant samples [35]. The recovery of the compounds in solvent is totally dependent on the solubility of the antioxidant compounds in solvents used for extraction. Thus, the polarity of solvents can increase the solubility of the antioxidant compounds [17]. In this study, three different methods have been used for the evaluation of the antioxidant capacity of the extracts namely Ferric Reducing Antioxidant Power assay (FRAP assay), DPPH free radical-scavenging assay and ABTS radical scavenging capacity.

Ferric-Reducing Antioxidant Power (FRAP) assay: In FRAP assay, the ferric ion of FRAP reagent is reduced to ferrous at low pH as a result of the activity of antioxidants present in the sample. The reduction of ferric iron produces intense blue color whose absorbance is measured at 593 nm [15]. The muskmelon pulp extracts showed the highest antioxidant activity as FRAP assay (Table 4) and lowest occurred in ash gourd while the watermelon pulp was higher in FRAP value than pumpkin pulp in most of the solvents except water and 100% acetone. The FRAP values of peels given in Table 5 showed that highest activity is in muskmelon extract followed by pumpkin while the ash gourd peel showed higher values than watermelon except in pure organic solvents i.e., ethanol, methanol and acetone. In case of pulps, 50% ethanol was the most effective solvent while 50% acetone had shown the highest antioxidant activity in peels. All the solvents showed significant difference (P<0.05) in their FRAP assay. The peel extracts contain high FRAP value than pulp extracts. This result is similar with other studies showing that the peels of different fruits have more antioxidant activity than pulp [35-37].

DPPH radical scavenging activity: DPPH assay is generally used for the estimation of free radical scavenging activity of the antioxidants. DPPH is stable at room temperature and produces a violet solution in solvent. Antioxidant compounds cause the discoloration of violet color to yellow color indicating the scavenging activity of the added

sample. This reduction results in loss of absorbance measured at 515 nm. The DPPH values of the extracts are presented in Tables 4 and 5 for pulps and peels respectively. The DPPH values for pulp was highest for muskmelon and lowest for ash gourd while the watermelon pulp was higher in DPPH value than pumpkin pulp in most of the solvents except 70% acetone, 70% methanol and 70% ethanol. For peels, highest activity was shown by muskmelon extract followed by pumpkin (except in water extract). The ash gourd peel extracts showed higher value

Solvents	Pumpkin			Ash gourd			Watermelon			Muskmelon		
	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)
Water 100	1.16 ± 0.2^{a}	14.87 ± 0.58^{a}	0.47 ± 0.08^{a}	0.96 ± 0.08^{a}	11.60 ± 1.01^{a}	0.34 ± 0.05^{a}	1.15 ± 0.14^{a}	19.53 ± 1.09^{a}	0.65 ± 0.09^{a}	2.17 ± 0.12^{a}	25.32 ± 0.86^{a}	1.05 ± 0.07^{a}
Acetone: Water												
100:0	1.83 ± 0.10^{bc}	28.68 ± 0.66^{b}	0.84 ± 0.1^{b}	1.13 ± 0.12^{bc}	24.10 ± 0.93^{c}	0.51 ± 0.07^{b}	1.84 ± 0.12^{b}	28.39 ± 1.15^{b}	0.84 ± 0.08^{bc}	3.02 ± 0.10^{c}	33.14 ± 1.22^{b}	1.54 ± 0.10^{b}
70:30	2.36 ± 0.09^{e}	37.67 ± 0.75^{d}	1.19 ± 0.05^{d}	1.26 ± 0.06^{cd}	31.64 ± 1.14^{e}	0.85 ± 0.09^{c}	2.44 ± 0.07^{d}	34.67 ± 1.29^{d}	1.36 ± 0.12^{d}	3.45 ± 0.11^{d}	45.16 ± 1.55^{d}	1.96 ± 0.15^{c}
50:50	2.84 ± 0.14^{g}	49.48 ± 1.10^{g}	1.45 ± 0.09^{e}	2.05 ± 0.10^{f}	41.37 ± 1.20^{h}	1.21 ± 0.13^{d}	3.37 ± 0.12^{f}	51.58 ± 1.08^{g}	2.03 ± 0.09^{f}	4.05 ± 0.13^{f}	57.05 ± 1.58^{e}	2.55 ± 0.12^{d}
Methanol: Water												
100:0	1.56 ± 0.15^{b}	28.31 ± 0.87^{b}	0.77 ± 0.09^{b}	0.99 ± 0.12^{ab}	21.77 ± 1.02^{b}	0.43 ± 0.07^{ab}	2.04 ± 0.07^{c}	29.44 ± 1.06^{b}	0.76 ± 0.06^{ab}	2.77 ± 0.11^{b}	35.30 ± 1.44b^{c}	1.14 ± 0.11^{a}
70:30	2.28 ± 0.14^{de}	38.56 ± 0.65^{de}	1.08 ± 0.12^{cd}	1.24 ± 0.08^{cd}	29.38 ± 1.08^{d}	0.75 ± 0.06^{c}	2.46 ± 0.10^{d}	36.44 ± 1.30^{d}	1.34 ± 0.09^{d}	3.13 ± 0.12^{b}	43.39 ± 1.10^{d}	1.49 ± 0.14b
50:50	2.66 ± 0.18^{fg}	47.64 ± 1.17^{f}	1.36 ± 0.07^{e}	1.77 ± 0.11^{e}	38.51 ± 1.13^{g}	1.12 ± 0.11^{d}	3.31 ± 0.13^{f}	48.52 ± 1.09^{f}	1.82 ± 0.09^{e}	3.84 ± 0.10^{e}	56.52 ± 1.56^{e}	2.41 ± 0.10^{d}
Ethanol: Water												
100:0	2.06 ± 0.13^{cd}	30.33 ± 1.08^{c}	1.04 ± 0.07^{c}	1.34 ± 0.11^{d}	25.52 ± 1.07^{c}	0.79 ± 0.05^{c}	2.17 ± 0.08^{c}	31.73 ± 0.94^{c}	0.96 ± 0.08^{c}	3.15 ± 0.11^{c}	36.53 ± 1.31^{c}	1.57 ± 0.09^{b}
70:30	2.52 ± 0.24^{ef}	39.68 ± 1.06^{e}	1.35 ± 0.08^{e}	1.95 ± 0.06^{f}	34.10 ± 1.14^{f}	1.09 ± 0.08^{d}	2.74 ± 0.10^{e}	39.58 ± 0.88^{e}	1.44 ± 0.1^{d}	3.54 ± 0.14^{d}	45.52 ± 0.94^{d}	1.95 ± 0.08^{c}
50:50	3.23 ± 0.18^{h}	51.16 ± 1.04^{h}	2.04 ± 0.09^{f}	2.26 ± 0.09^{g}	47.11 ± 1.39^{i}	1.54 ± 0.10^{e}	3.45 ± 0.07^{f}	52.53 ± 1.21^{g}	2.24 ± 0.10^{g}	4.21 ± 0.12	63.29 ± 2.46^{f}	2.78 ± 0.14^{e}

All values are means ± standard deviations of data from three independent experiments
Different superscripts (a, b, c, d…..i) in the same column indicate significant difference (P<0.05)

Table 4: Antioxidant activities (obtained from FRAP, DPPH and ABTS assay) in pulp of four cucurbits using different solvents.

Solvents	Pumpkin			Ash gourd			Watermelon			Muskmelon		
	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)	FRAP (µM of Fe(II) per g)	% DPPH	ABTS (µmol Trolox/g)
Water 100	2.14 ± 0.09^{a}	24.23 ± 1.14^{a}	1.25 ± 0.12^{a}	1.77 ± 0.18^{a}	19.55 ± 0.70^{a}	0.99 ± 0.05^{a}	1.18 ± 0.10^{a}	25.28 ± 1.23^{a}	0.86 ± 0.09^{a}	2.92 ± 0.15^{a}	32.52 ± 1.22^{a}	1.15 ± 0.08^{a}
Acetone: Water												
100:0	3.25 ± 0.10^{c}	40.50 ± 1.25^{c}	1.80 ± 0.05^{c}	2.28 ± 0.10^{c}	36.16 ± 0.90^{d}	1.26 ± 0.08^{b}	2.38 ± 0.12^{c}	32.82 ± 1.09^{b}	1.27 ± 0.08^{b}	3.57 ± 0.11^{b}	43.82 ± 1.08^{c}	1.77 ± 0.09^{b}
70:30	4.36 ± 0.07^{f}	49.48 ± 1.08^{e}	2.24 ± 0.13^{ef}	3.33 ± 0.10^{e}	42.58 ± 1.03^{f}	1.74 ± 0.11^{c}	3.13 ± 0.09^{e}	42.67 ± 0.69^{d}	1.66 ± 0.07^{c}	4.70 ± 0.07^{d}	52.54 ± 1.07^{e}	2.44 ± 0.08^{d}
50:50	5.14 ± 0.13^{i}	64.79 ± 1.90^{g}	3.11 ± 0.07^{h}	4.21 ± 0.16^{g}	60.56 ± 1.10^{i}	2.54 ± 0.10^{e}	3.86 ± 0.13^{g}	58.23 ± 0.89^{g}	2.46 ± 0.10^{e}	5.47 ± 0.10^{g}	68.04 ± 1.41^{g}	3.37 ± 0.11^{f}
Methanol: Water												
100:0	3.13 ± 0.09^{bc}	34.57 ± 1.71^{b}	1.34 ± 0.10^{b}	2.03 ± 0.07^{b}	30.25 ± 0.76^{b}	1.06 ± 0.05^{a}	2.13 ± 0.09^{b}	31.31 ± 1.28^{b}	0.96 ± 0.10^{a}	3.38 ± 0.12^{b}	37.99 ± 1.71^{b}	1.63 ± 0.09^{b}
70:30	3.85 ± 0.14^{d}	43.79 ± 1.26^{d}	1.97 ± 0.08^{d}	2.88 ± 0.11^{d}	40.72 ± 1.10^{e}	1.64 ± 0.09^{c}	2.73 ± 0.10^{d}	39.31 ± 1.02^{c}	1.19 ± 0.12^{b}	4.20 ± 0.10^{c}	47.11 ± 1.37^{d}	2.13 ± 0.08^{c}
50:50	4.65 ± 0.15^{g}	59.29 ± 1.05^{f}	2.35 ± 0.15^{f}	3.70 ± 0.07^{f}	54.63 ± 0.87^{g}	2.15 ± 0.05^{d}	3.55 ± 0.13^{f}	51.91 ± 1.55^{e}	2.14 ± 0.10^{d}	5.00 ± 0.11^{e}	62.73 ± 1.28^{f}	2.58 ± 0.10^{d}
Ethanol: Water												
100:0	2.96 ± 0.09^{b}	38.57 ± 1.16^{c}	1.46 ± 0.11^{b}	2.19 ± 0.07^{bc}	32.68 ± 1.20^{c}	1.14 ± 0.08^{ab}	2.26 ± 0.08^{bc}	32.50 ± 0.97^{b}	1.23 ± 0.10^{b}	3.43 ± 0.07^{b}	41.89 ± 1.04^{c}	1.67 ± 0.09^{b}
70:30	4.04 ± 0.11^{e}	47.38 ± 1.11^{e}	2.08 ± 0.09^{de}	3.28 ± 0.06^{e}	42.14 ± 0.95^{ef}	1.66 ± 0.12^{c}	2.77 ± 0.10^{d}	41.63 ± 0.92^{d}	1.55 ± 0.09^{c}	4.38 ± 0.11^{c}	50.80 ± 1.11^{e}	2.16 ± 0.07^{c}
50:50	4.85 ± 0.12^{h}	61.24 ± 1.21^{f}	2.65 ± 0.06^{g}	3.88 ± 0.08^{f}	56.40 ± 1.36^{h}	2.25 ± 0.07^{d}	3.63 ± 0.15^{f}	55.60 ± 1.18^{f}	2.29 ± 0.04^{d}	5.22 ± 0.16^{f}	64.44 ± 1.17^{f}	2.84 ± 0.11^{e}

All valuesare means ± standard deviations of data from three independent experiments
Different superscripts (a,b,c, d…..h) in the samecolumn indicate significant difference (P<0.05)

Table 5: Antioxidant activities (obtained from FRAP, DPPH and ABTS assay) in peel of four cucurbits using different solvents.

than watermelon except for extracts in water, 70% acetone and 100% methanol. All the solvents showed significant difference (P<0.05) for their DPPH free radical scavenging activity. The similar trend was obtained as in polyphenols, the extracts in 50% ethanol and 50% acetone showed the highest antioxidant activity in pulp and peel respectively.

Antioxidant capacity determined by radical cation ($ABTS^{\cdot+}$): ABTS assay is based on the reaction of the $ABTS^{\cdot+}$ radical cation generated in the assay with the antioxidant present in the sample. This method takes comparatively less time than the other methods and it is also used to confirm the result obtained with DPPH, as both are similar in their antioxidant mechanism. The result showed that all the fruit pulps and peels exhibit the antioxidant capacity but in different degrees. Table 5 showed that muskmelon peel had the highest activity than pumpkin peel except for water and 100% acetone extracts. After these the ash gourd shows higher value than watermelon peel extracts except in 100% acetone, 100% ethanol and 50% ethanol. In case of pulp extracts, muskmelon pulp showed highest antioxidant activity followed by watermelon. The watermelon extract showed higher antioxidant value than pumpkin in most of the solvent extracts and ash gourd had given the lowest ABTS value. The result showed the similarity with the result of DPPH i.e., the pulp extracts showed highest antioxidant activity in 50% ethanol extract while the peel extracts in 50% acetone.

These results indicated that the TPC, TFC, FRAP, DPPH and ABTS values were susceptible to solvents used for extraction. The pure solvents, acetone 100% and ethanol 100% showed comparatively similar results in extraction efficiency with most of the samples followed by methanol, and water, respectively. Aqueous organic solvent mixture gave the highest values. 50% ethanol and 50% acetone were the best solvents to obtain extracts with higher quantity of polyphenolic content and antioxidant activities. In most of the cases, these two solvents showed significant difference (P<0.05) for all the samples. The value of the antioxidant activities varies in different extracts which might be related to the change in the polarity of different solvents [21]. From the results obtained, it may be suggested that the change in the polarity of organic solvent by addition of water (up to 50%) possibly enhance the extraction of antioxidant compounds. The results also indicated that the polyphenolic content and antioxidant activity of the peels was more than the pulps. The majority of fruit peels exhibit high antioxidant activity than pulp [35]. The antioxidant activity of the fruits might be influenced by its geographical location, types of cultivar, harvest season and storage conditions [38].

Correlation analysis between polyphenolic content and antioxidant activity

Despite of the different fruit, correlation analysis (Table 6) was performed between polyphenolic content and antioxidant activity among all pulp and peel extracts for each solvent. The extracts from 10 different solvents exhibited significant linear correlation (P<0.01) amongst all the parameters tested namely TPC, TFC, FRAP, DPPH and ABTS. The correlation coefficient (r) between TPC and TFC from different solvent extracts, it was shown that TPC and TFC had the similar trend with all the solvent exhibiting high linear correlation coefficient ($r \geq 0.95$). The result also signified a strong correlation between total phenolic content and FRAP assay showing similarity with the correlation found by Benzie and Stezo [16]. In case of correlation between TPC and FRAP, and TFC and FRAP almost a similar trend is observed showing the highest correlation in 50% acetone (r=0.961, 0.938, respectively) and the lowest was observed in 100% ethanol (r=0.792, 0.781, respectively). The correlation between TPC and DPPH, and TFC and DPPH, the highest correlation was in 50% acetone (r=0.959, 0.936, respectively) and lowest in 100% methanol (r=0.796, 0.772, respectively). Between TPC and ABTS, and TFC and ABTS, the highest correlation in 50% acetone (r=0.948) and 70% methanol (r=0.906) respectively, and the lowest was observed in 100% ethanol (r=0.794) and 50% methanol (r=0.761) respectively. These correlations specifies that higher the polyphenolic value, higher the antioxidant activities confirming that polyphenolic compounds are the main components that contribute to the antioxidant activities of these fruits.

Correlation analysis between the antioxidant activities showed significant linear correlation (P< 0.01) in all the solvent extracts. FRAP, DPPH and ABTS follow the same mechanism of single electron transfer (SET) in which it identify the capability of the prospective antioxidant for transferring of single electron for reduction of any compound. Both Fe (III)-TPTZ and $ABTS^{\cdot+}$ have similar redox potential of less than 0.7 V. The conditions of reaction for maintaining the iron solubility differ in both the methods as ABTS assay is done at neutral pH while FRAP assays is carried out at acidic pH 3.6. Thus, the values obtained by both may be comparatively relative [36]. ABTS assay is used to confirm the results obtained by DPPH assay. This shows that all the methods are correlated to each other. The correlation coefficient between FRAP and DPPH, and FRAP and ABTS, the highest value was shown in 50% acetone (r=0.971, 0.977, respectively) and the lowest was observed in water (r=0.813, 0.802, respectively). The correlation between DPPH and ABTS, the highest correlation was in 50% acetone as well as 70% acetone (r=0.963) while lowest occurred in water (r=0.827). Between ABTS and Reducing Power, the highest correlation was in 70% acetone (r=0.950) while lowest occurred in water extract (r=0.827). Among all the solvents used for extraction, 50% acetone showed higher correlation coefficient between most of the assays.

Conclusion

The finding of our study revealed that the extracts of the selected cucurbits have shown the significant antioxidant activity depending upon the type of solvent used for extraction. Acetone was found to be the best solvent followed by ethanol, methanol and water respectively. The mixture of organic solvents and water enhances the efficiency of extraction by making both lipophilic and hydrophilic compounds soluble in the mixture. The 50% aqueous acetone was the most effective solvent while water was found to be the least effective for all extracts. The 50% acetone showed the highest extraction in case of peels while 50% ethanol in case of pulp. The correlation between the polyphenolic content and antioxidants was high for all the extracts. The highest correlation coefficient between various assays was found in 50% acetone indicating it as the best solvent for extraction of polyphenolic compounds in these cucurbits. The phenolic content, flavonoid content, and the antioxidant activity were highest in muskmelon fruit when compared with other three fruits. Thus, the work indicated that these fruits are good source of phytochemicals that can be extracted by using a proper solvent system.

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Correlation coefficient (r)	TFC	FRAP	DPPH	ABTS
100% Water extract (N=24)				
TPC	0.953	0.801	0.926	0.795
TFC	-	0.879	0.921	0.861
FRAP	-	-	0.813	0.802
DPPH	-	-	-	0.827
100% Acetone extract (N=24)				
TPC	0.968	0.822	0.954	0.858
TFC	-	0.815	0.932	0.864
FRAP	-	-	0.910	0.965
DPPH	-	-	-	0.913
70% Acetone extract (N=24)				
TPC	0.984	0.913	0.892	0.885
TFC	-	0.911	0.893	0.878
FRAP	-	-	0.970	0.970
DPPH	-	-	-	0.963
50% Acetone extract (N=24)				
TPC	0.976	0.961	0.959	0.948
TFC	-	0.938	0.932	0.902
FRAP	-	-	0.971	0.977
DPPH	-	-	-	0.963
100% Methanol extract (N=24)				
TPC	0.975	0.871	0.796	0.924
TFC	-	0.864	0.772	0.897
FRAP	-	-	0.947	0.931
DPPH	-	-	-	0.891
70% Methanol extract (N=24)				
TPC	0.986	0.895	0.802	0.914
TFC	-	0.888	0.791	0.906
FRAP	-	-	0.950	0.948
DPPH	-	-	-	0.867
50% Methanol extract (N=24)				
TPC	0.981	0.914	0.882	0.813
TFC	-	0.898	0.862	0.761
FRAP	-	-	0.962	0.927
DPPH	-	-	-	0.929
100% Ethanol extract (N=24)				
TPC	0.977	0.792	0.882	0.794
TFC	-	0.781	0.874	0.765
FRAP	-	-	0.966	0.934
DPPH	-	-	-	0.907
70% Ethanol extract (N=24)				
TPC	0.974	0.908	0.860	0.854
TFC	-	0.876	0.838	0.841
FRAP	-	-	0.958	0.949
DPPH	-	-	-	0.946
50% Ethanol extract (N=24)				
TPC	0.955	0.937	0.834	0.800
TFC	-	0.915	0.779	0.765
FRAP	-	-	0.921	0.919
DPPH	-	-	-	0.922

Correlation is significant at the 0.01 level (2-tailed)

Table 6: Correlations between phenolic contents and antioxidant activities of various solvent extracts.

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Fibrous Cellular Structures are Found in a Commercial Fruit Smoothie and Remain Intact during Simulated Digestion

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Abstract

The intactness of cell wall structures in foods has important repercussions for nutrient digestion and availability. In this study, we show the presence of intact cell wall structures in a commercial fruit smoothie (blend of banana, mango, orange and apple) and fruit purée (banana, mango), but not in fruit juices (apple or orange). Small clusters of cells were observed in fresh crushed fruit (banana, mango, and apple), the size of the cluster dependent on the type of fruit. When the smoothie was subjected to simulated gastro-intestinal digestion, cell wall structures were found abundantly even after 16 hrs of agitated incubation with digestive enzymes (protease, amylase and amyloglucosidase). Total dietary fibre (TDF) content of the smoothie was measured using the AOAC (991.43) and integrated fibre (IF) analysis methods. TDF-AOAC value was significantly lower (1.61%) than the TDF-IF (2.22%), but the ratio of insoluble to soluble dietary fibre (IDF: SDF) was consistently 1:3. Disruption of the cell wall structures in the smoothie by high shear homogenisation led to a 68% reduction in viscosity, 30% reduction in TDF content and a 10% increase in SDF. These experiments suggest that cell wall structures similar to those observed in crushed fruit are preserved during commercial smoothie manufacture and are retained during digestion. Their presence may have implications for fibre quantification and fibre functionality in the gut. We discuss the need to consider fibre structure, as well as content, when evaluating the nutritional properties of fruit and their products.

Keywords: Soluble dietary fibre; Insoluble dietary fibre; Cell wall; Fruit smoothie; Simulated digestion; Cellular structures; INFOGEST

Abbreviations: AOAC: Association of Official Analytical Chemists; IF: Integrated Fibre; IDF: Insoluble Dietary Fibre; SDF: Soluble Dietary Fibre; TDF: Total Dietary Fibre

Introduction

Consumption of fruit and fruit products has been associated with a reduced risk of overall mortality and some non-communicable diseases, leading to intake recommendations of least 3 to 4 portions of fruit a day for the general population [1,2]. The protective effect is reduced, but not eliminated, when the fruit is in the juice form, indicating that cellular structure may be important for health beneficial effects. The intactness of cell wall structures has been shown to regulate starch digestion [3,4] and the availability of iron [5] in cooked legumes, and the rate of lipolysis in nuts [6,7]. Intact cellular structures and cell clusters were also observed following chewing of mango and banana fruits [8]. These cellular structures remained visually intact during *in vitro* colonic fermentation with smaller particles (<1 mm) fermenting more rapidly that larger ones. In contrast, fermentation of carrot cell clusters showed that particles measuring approximately 150-300 μm were more rapidly fermented than smaller clusters or single cells [9]. These studies suggest that the intactness of cellular structures may affect fruit and vegetable functionality in the gut. Fleshy fruit tissue is made up largely of parenchyma-type cells with thin, elastic cell walls. These cells are inter seeded with rigid vascular fibres. The Total Dietary Fibre (TDF) content of fleshy fruit varies considerably depending on species and ripening stage. Apples, bananas, mangoes and oranges have reported TDF values of 2.21%, 1.79%, 1.76% and 2.35% respectively while orange juice has a reported TFD value of only 0.30% [10]. Food processing can affect fibre content and properties [11]. For example, the solubility of fibre from a range of legumes was increased following canning [12]. Fruits tend to have higher soluble dietary fibre (SFD) to insoluble dietary fibre (SDF: IDF) ratio of around 1:2, compared to cereals (1:3) and legumes (1:4) [10,12]. While consumption of whole fruits would ensure ingestion of cellular structures, food consumption data indicates that around two thirds of adults do not consume enough fruit (Public Health England, 2016). Fruit beverages such as smoothies have been suggested as convenient and cost effective foods that provide similar nutrient and functionality to whole fruit [13]. Fruit smoothies are blended fruit products, characterised by a viscous consistency compared to fruit juices, though the viscosity varies greatly amongst products. Despite the popularity of smoothies since the 1990's, the fibre properties of smoothies have not been well characterised physically, chemically or biologically. The effect of food processing on cellular structures also needs further investigation.

The analysis of TDF, SDF and IDF is problematic due to the diverse nature of fibre components. The widely used Association of Official Analytical Chemists fibre analysis protocol (AOAC method 991.43) generally includes homogenisation and high temperature amylase digestion steps [14] both of which may affect fibre content and solubility. More recently, protocols using lower incubation temperatures with pancreatic enzymes have been suggested, but not widely adopted [15,16]. Recently, a harmonised *in vitro* simulated digestion protocol was described by the INFOGEST consortium [17]. This method is widely used to investigate protein and lipid digestion, but has not yet been used in the context of fibre analysis. It has been recognised that typical methods of fibre analysis are not likely to reflect physiological function [18]. We postulate that cell wall structure has an effect on fibre measurement.

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The aim of this study was to observe whether cell wall structures are present in a commercial fruit smoothie as well as two purées (banana and mango) and two juices (apple and orange), and to compare those structure to those found in crushed fruit (apple, banana, mango). The TDF, SDF and IDF content of the smoothie and juices was measured using the standard AOAC method 991.43 and the more gentle Integrated Fibre (IF) method. We used the INFOGEST protocol to assess the intactness of cell wall structures after digestion. To assess the role of cell wall structure on fibre functionality, the effect of high-shear homogenisation on viscosity, fibre content and solubility was evaluated. We discuss the need to consider fibre structure, as well as content, when evaluating the health benefit of fruit and their products.

Materials and Methods

Food materials

Fruit smoothie (Magnificent Mango), orange and apple juice, banana and mango purées were all from Innocent Ltd (UK). Ripe fresh fruit (banana, apple, mango and orange) was purchased from a local store. Smoothie samples were homogenised using a Polytron homogeniser (10,000 rpm for 2 min, 5 min, and 10 min). Fruit samples (10 g) were crushed using a miniature extrusion Ottawa cell in TA.XT Texture Analyser (Stable Microsystems) using a compression speed of 2 mm s^{-1}. Crushed fruit was recovered and used immediately for microscopic observation.

Determination of TDF, SDF and IDF

Two protocols were used to determine the TDF, SFD and IDF content of the smoothie and the juices. Firstly, the AOAC method 991.43 was used using a total dietary fibre kit (K-TDFR) from Megazyme (Bray, Ireland) with few modifications, namely 5 g of smoothie, juice or fruit were used instead of 1 g; the fritted glass filters were replaced with three layers of glass filters (GFA, Whatman) placed on ceramic filters. Secondly, the integrated total dietary fibre kit (K-INTDF) from Megazyme (Bray, Ireland) was used, using similar modifications as above. SDF and IDF were separated by ethanol precipitation as per the kits' instructions, except a high speed centrifugation (47,000 × g, 4°C, 60 min) step was included to facilitate the separation of the SDF prior to filtration.

Digestion with INFOGEST protocol

The INFOGEST protocol was applied to the smoothie sample (5 g) according to the protocol [17] except that the oral phase was omitted. At the end of the digestion, the supernatant was observed under the microscope. The undigested residue was collected by filtration as described above. All fibres were dried at 60°C and cooled in a desiccator before weighing.

Microscopy

An aliquot of smoothie, juice or digested fractions was diluted 1:10 with 1.5 N NaOH (w/v) containing 0.1% (w/v) toluidine blue and 0.25% (w/v) calcofluor white (Fluorescent Brightener 28, both stains from Sigma-Aldrich, UK) and incubated for 10 min. Suspensions were directly placed onto poly lysine covered glass slides, covered with a glass cover slip and observed under an inverted microscope equipped with UV fluorescence (Olympus-BH2). Crushed fruit were suspended in water and stained as described.

Viscosity determination

The viscosity of the smoothie and juices were determined using a Bohlin C-VOR Shear Rheometer with an axial geometry, at minimum and maximum shear rates of 0.1 and 200 l s^{-1}.

Results

Presence of intact cell wall structures in crushed fruit, purées and smoothie but not in juices

Fruit was crushed using an extrusion probe designed to simulate the chewing process in the mouth. Crushed fruit contained clusters of intact cells with smaller clusters observed in mango (<10 cells/cluster) followed by banana (<15-20 cells/cluster) and apple (>50 cells/cluster). Crushed apple had broken cells at the periphery of the cluster indicating cell breakage and stronger cell adhesion in apple compared to the other fruits. Starch granules could be clearly observed within banana cells, but not in mango or apple cells Calcofluor white staining showed that the structures contained cellulosic material (Figures 1A-1F). Cellular structures similar to those present in fruits were observed in the banana purée (Figures 2A and 2B). In the mango purée, stringy fibre like structures were observed which looked like disrupted cells (Figures 2C and 2D). In juices, only scattered stringy ribbons, presumed to be of vascular origin, and were observed (Figures 2E-2 H).

Intact cellular structures were also found suspended in the smoothie with a calculated density of 3300 cells per mL (Figure 3A and 3B). The cell structures were generally single cells of elongated oval shapes with a range of sizes from 50 to around 400 µm in diameter. Toluidine blue staining revealed cytoplasmic content within the cells, but no obvious starch granules (Figure 3A). The results suggest that commercial smoothie processing preserves the cellular structures of fruits.

High-sheer homogenisation for 10 min of the smoothie led to a marked reduction in calcofluor staining and a disruption of the cellular structures (Figures 3C and 3D). The observed ribbon-like structures were similar to those found in mango purée (Figures 2C and 2D). Following simulated digestion of smoothie, cellular structures were abundantly present, even after 16 hrs of gastro-intestinal digestion (Figures 3E and 3F). These results support previous observations for chewed fruit [8] and suggest that fruit parenchyma cells withstand human digestive processes. It is likely, as was observed by Low et al., that the smoothie structures would be fermented by colonic bacteria.

Determination of TDF, SFD and IDF content of smoothie and juices

The TDF content of commercial smoothie, as well as two fruit juices, were determined using two fibre protocols (Table 1). The smoothie AOAC-TDF content was around 30% lower level than when the IF method was used ($p<0.05$). The AOAC 991.43 method includes a 30 min high temperature heating step (100°C) which may destroy the cellular structure. Heating of potato tubers using different processing methods led to separation of cells, but maintenance of cell wall integrity [19]. The IF method which uses pancreatic amylase at 37°C is more representative of physiological conditions and likely to preserve fibre cellular structures (as supported by observation of intact cellular structure following the INFOGEST protocol shown in Figures 3E and 3F.

Homogenisation significantly decreased the TDF content of the smoothie by about 10% ($p<0.05$) using both methods. As seen in Figures 3C and 3D, homogenisation destroys the cellular structures and also results in some fibre loss. However, it appears that homogenisation affects fibre content less than heating. There was moderate positive association between viscosity and fibre content ($R^2=0.7136$).

The proportion of soluble to insoluble fibre was calculated (Table 1). In smoothie, SDF and IDF made up 65 and 35% of the total fibre

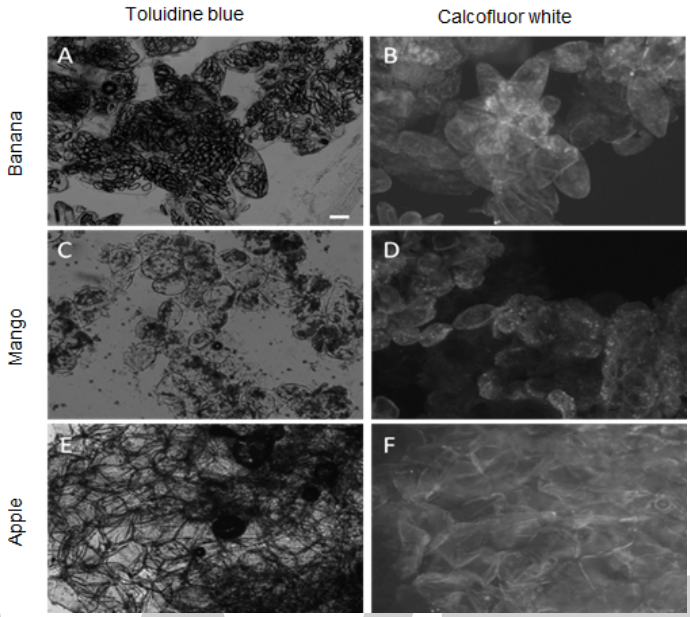

Figure 1: Light and UV microscopy images of crushed fruits. Cellular contents were stained with toluidine blue (A, C, E) revealing abundant starch granules in banana (A) and to a lesser extent in mango (C), but not in apple (A). Cell walls were stained with calcofluor white (A, D, F) showing cellular clusters in all fruits. Scale bar=10 µm.

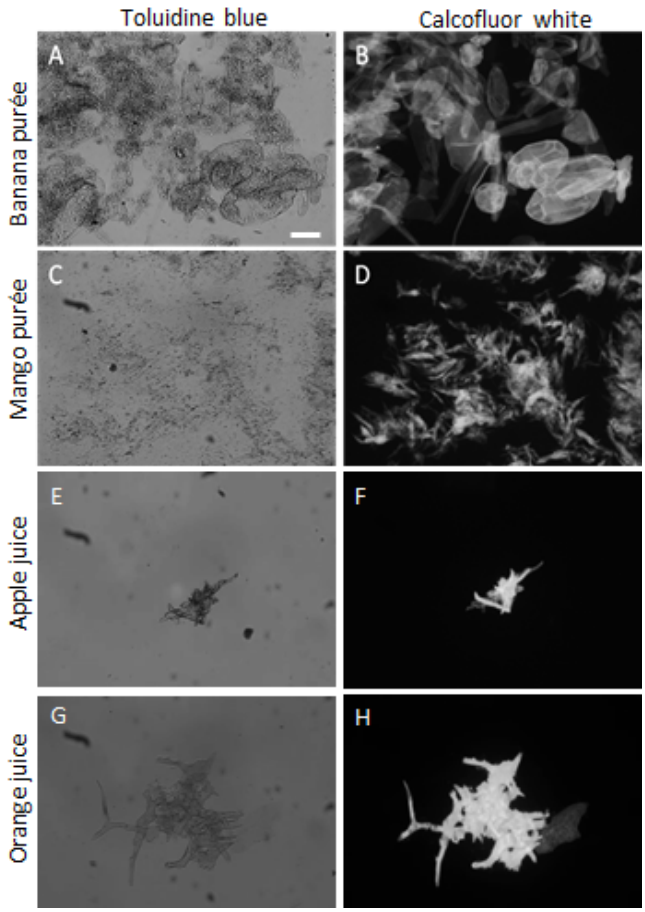

Figure 2: Light and UV microscopy images of puréed or juiced fruits. Cellular contents were stained with toluidine blue (A, C, E, and G), there were no apparent starch granules stained. Cell walls were stained with calcofluor white (B, D, F, and H) showing cellular structures in the banana puree (B) but not in mango purée (D), apple (F) or orange juice (H). Scale bar=10 µm.

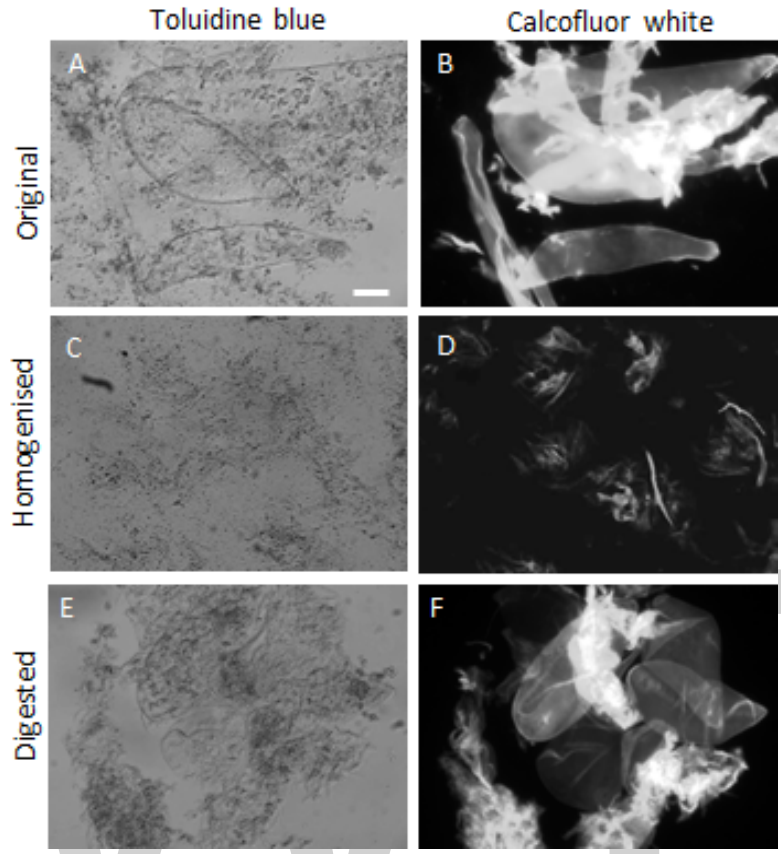

Figure 3: Light and UV microscopy images of commercial smoothie sample. Original smoothie, homogenised and digested smoothie were stained with toluidine blue (A, C, E) to reveal cellular contents. No starch granules were identified. Cell walls were stained with calcofluor white (B, D, and F). Intact cellular structures were observed in original and digested smoothie, but not in the homogenised sample. Scale bar=10 μm.

	TDF		SDF	IDF	IDF:SDF
	AOAC	Integrated	AOAC	AOAC	AOAC
Smoothie (Original)	1.6 (0.1)^{a}	2.2 (0.3)^{d}	1.2 (0.1)^{b}	0.4 (0.1)^{d}	1:3
Smoothie (Homogenised)	1.2 (0.1)^{b}	1.6 (0.3)^{a}	1.0 (0.1)^{c}	0.2 (0.1)^{e}	1.5
Orange Juice	0.9 (0.1)^{c}	1.2 (0.2)^{b}	0.8 (0.1)^{c}	0.2 (0.1)^{e}	1:4
Apple Juice	0	0	ND	ND	ND

Table 1: Total dietary fibre (TDF) analysed using two different analytical methods, the AOAC (991.43) and integrated protocol. Soluble dietary fibre (SDF) and insoluble dietary fibre (IDF) measured with the AOAC method and ratio of IDF:SDF. Values show mean of at least three replicate determinations and standard deviation of the mean in parentheses. Different letters show significant different values according to one factor ANOVA (95% confidence). NA: Not applicable; ND: Not determined.

content respectively (ratio SDF: IDF of 3:1). The proportion of SDF is higher than what is reported for whole fruit (SDF:IDF of 1:2) [10]. SDF increased significantly with homogenisation time to account up to 75% of total fibre after 10 min of homogenisation. In summary, cell wall structures were observed in all crushed fruits, some purées and the smoothie sample. Structures are preserved during simulated digestion. Homogenisation disrupts the cellular structure, reduces fibre content and viscosity.

Discussion

The importance of food structure for health functionality is increasingly being recognised [20]. Cell walls are natural structures that occur in plant foods that offer a natural barrier to molecular exchange and enzymatic digestion of cellular contents [18]. Most studies on cellular structures have focused on legumes and nuts which have relatively thick and rigid cell walls [21]. These act as physical barriers to digestion and nutrient release. In fruits, cell walls are relatively thinner, but appear remarkably resilient. Potato tubers have thin parenchyma-type cell walls which appear to resist heat processing, but do not appear to restrict digestion of starch within the cells [19]. Fruit cellular structures have been observed after chewing of banana and mango fruits [8] and these structures were largely preserved during *in vitro* digestion. The preservation of the cellular structures in processed fruit products has not been previously shown. In the present study, we show that a commercial sample of smoothie has a high concentration of apparently intact fruit cells which are also preserved during digestion. This indicates that smoothie processing would offer similar benefits to whole fruit to consumers. The health implications of these structures is not yet clear but they may contribute to the physiological benefits of plant foods, independent of total fibre content. These effects may partly explain why fruit juices offer lower disease protection compared to whole fruits, even though the chemical composition is very similar. Most dietary surveys do not have enough categorisation of fruit products and this has been suggested as an issue in not only measuring what people consume but also in formulating clear nutritional advice [22]. The physical properties of fibre are important for its function. Yet, the analytical techniques used to quantify fibre content for composition tables and epidemiological studies are methods that destroy these

physical characteristics and may not give a physiological indication of fibre functionality. We suggest that epidemiological and interventional studies consider fibre structure, as well as content and solubility, when evaluating the nutritional properties of fruit and their products.

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Toxins, Malnutrition, Stress, Infections and Electromagnetic Pollution: Looking about New Perspectives in Development of Diseases

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Abstract

This review addresses the effect of ingested toxins, malnutrition, stress, infection, and electromagnetic pollution in development of diseases. Some toxins are transmitted by umbilical cordon blood in birth and others are ingested by orally as monosodium glutamate, fructose, soft drinks, gluten, oils, xenoestrogens and heavy metals. Consequently, these toxins if accumulate in the body and overloading the liver, favoring only diseases. Lack of basic nutrients as water, magnesium, iodine, stomach acid, amino acids and fatty acids were strongly linked with achlorhydria, growth of diabetes and cardiovascular disease. Dental infection destroys mitochondria's through of gliotoxins, mercury, thioesters and oral infections can cause many systemic diseases. Root canals contain a significant source of bacteria and fungi in the circulation blood and endodontic treatment can be the cause of anaerobic bacteremia and fungemia predisposing the chronic disease. Sleep deprivation and to sleep with light on, tablets, cell phone next to bed commits the release of melatonin by pineal gland. Electromagnetic pollution contribute for headaches, depression, anxiety, palpitation and these symptoms are linked with electrical hypersensitivity that have been associated with diabetes, multiple sclerosis and attention deficit hyperactivity disorders. Thereby, we believe that is need paradigm change in the medical model for investigate these factors in your patients because currently this is not being done.

Keywords: Toxins; Malnutrition; Stress; Infections; Electromagnetic pollution

Introduction

Nowadays, why do some people get sick and others not? Worldwide, the people live connected the internet, drinking more beer and sugar-sweetened beverage that water, consuming more processed foods compared the ancestors, do not sleep needed and taking drugs for control some diseases. Hippocrates, father of medicine, 431 B.C. said: Let food be thy medicine and medicine be thy food, however, currently we do drugs our food and drugs are foreign substances for human body containing adverse effects. Unfortunately, current medical model is based in suppression of symptoms thereby, are prescribed more drugs for treatment of symptoms. On the other hand, the conceit of prevention of diseases and like having health is little taught in majority medical schools worldwide, and this form, the physicians become experts only in diseases treatment.

When compared Neolithic Era with lifestyle current is noteworthy the differences in the type of food, time of sun exposure, hours of sleep, physical activity (Table 1). We consumed more calories daily compared decade of 1980 modifying the leptin, hormones satiety [1,2].

Therefore, is crucial clarify the factors that impart resilience or contribute development some diseases that may arise through of toxins, malnutrition, infections, stress and electromagnetic pollution (Figure 1). Thereby, is objective this narrative review explain role these factors in diseases source and suggest alternative measures for prevention and illness treatment.

Toxins

We eat what not should eat and thus overload the liver that is responsible for cleaning of toxins consumed as sugar, some sweeteners and oils for cooking, soft drinks and fructose (Figure 2). Toxins have presents in air, soil, water and some can be in the foods. However, some toxins can be transmitted umbilical cordon blood of according with Environmental Working Group (EWG). This study, the researchers found an average 200 industrial chemicals and pollutants in umbilical cord blood from 10 babies born in August and September 2004. After cord was cut, umbilical cord blood harbored pesticides, gasoline, consumer product ingredients, garbage and waster from burning coal [3]. High intake of sugar in pregnancy is great problem for newborns. Results of study HAPO (Hyperglycemia and Adverse Pregnancy Outcomes); found that of higher levels maternal glucose were associated with birth weight and an increased in the levels C-peptide in the cord-blood [4].

Pesticides are considered essential for development of agriculture in worldwide, moreover the high levels of pesticides accepted for some countries like Brazil, become a great problem of health public [5]. Furthermore, they kill the funds and decreased the production of humic acid and fulvic acid which are crucial in the formation of feedstock for soil. Atrazine is the one of the most widely used herbicides in the Unites State in cultivation and have been associated with complications in female reproduction [6,7] and toxicity on human liver [8]. Heated Foodstuffs are dangerous for health. Besides, is noteworthy that French fries are strongly consumed for children's, teens and adults the entire world, but when the potato is heated, she becomes in acrylamide, a carcinogen compound that may build-up in body human [9]. Thereby, there is a toxic overload in liver, kidneys, gut, pancreas and immune dysregulation contributing for appearance of diseases [10].

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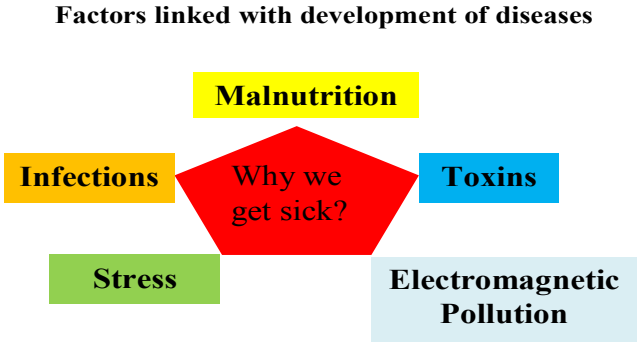

Figure 1: Ingested of toxins, more the malnutrition, stress, infections and electromagnetic pollution can contribute in development of diseases.

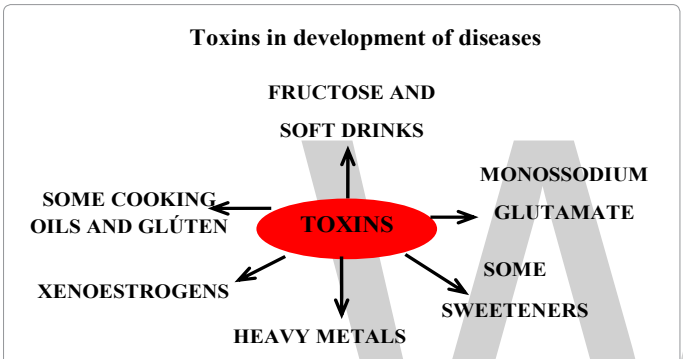

Figure 2: Exhibition to xenoestrogens and heavy metals added high consumption of fructose, monosodium glutamate, gluten and some cooking oils can contribute in development of chronic diseases.

Monosodium glutamate (MSG) is the sodium salt of glutamic acid used in the food industry as flavor enhancer that intensifies for meaty, stews and meat soups that may increase sensibility for the flavor umami. MSG produces a salty flavor when added to food, thus excites your taste buds umami stimulating the release of brain neurotransmitters as glutamate. Glutamate is a neurotransmitter excitatory essential role for learning and memory it being, crucial for body in the produce of GABA (gamma-aminibutyric acid), which is a calming and inhibitory brain chemical. However, high intake of processed foods with MSG may make body lose the ability to convert glutamate into GABA, leading the build-up of glutamate with deficiency of GABA. Therefore, these dates has been linked to serious disorders, like: Obesity and Metabolic Syndrome [11,12], Autism [13] and Alzheimer's disease [14].

Artificial sweeteners are consumed frequently for people trying to lose weight including obese children, patients with Diabetes and some sweeteners can have negative effects. A study done in 2014 found that consumption of artificial sweeteners it is associated with development of glucose intolerance through of induction of compositional and functional alterations to the intestinal microbiota in mice [15]. Aspartame is artificial sweeteners composed of methanol (10%) phenyalanine (50%) and aspartic acid (40%). After, human body convert methanol in formate, it can be excreted or can give rise to formaldehyde and a number of other highly toxic derivatives as the diketopiperazine, a carcinogen [16]. In a review done in 2008 researches reported that aspartame altered amino acid metabolism, integrity of nucleic acids, neuronal function, and balance endocrine and brain concentration catecholamines [17]. Furthermore, previously studies suggested an association between Aspartame consumption and risk of type 2 Diabetes [18] and preterm delivery [19].

Fructose is a kenotic monosaccharide sweeter that glucose or sucrose. Your absorption, digestion, metabolism differs from those of glucose. Unlike glucose, she does not stimulate insulin secretion or enhance leptin productions that control the food intake and body weight. Thus the high fructose consumption may contribute to increase energy intake and obesity [20]. There was an increase significantly of intake fructose of 1970 to 2000 and a 25% increase in added sugars this period [21]. In the pre-industrialization, the average consumption was day 15 g/fructose day and we obtained fructose through fresh fruits, vegetables and honey. However, with the inclusion of high-fructose corn syrup (HFCS), a sweetener made from corn starch more cheap that white sugar and done for replacing table sugar in food, made with that fructose intake increased 37 g/day and currently average consumed is 72.8/day [22]. Consequently, there was an increase of diseases as metabolic syndrome [23], diabetes [24], hypertriglyceridemia [25], nonalcoholic fatty liver disease [26] and cardio metabolic diseases due to high intake of HFCS. We increased sugar and fructose intake in the foods producing a new disease for society, the diabesity [27].

The soft drinks intake has been linked the type 2 diabetes [28,29], risk of gout [30], obesity and metabolic syndrome [31], deleterious effects on bone health [32] and coronary artery calcification [33]. Beyond sodium, some soft drinks containing caffeine which has diuretic effect, this form, how much more caffeine and sodium have in soft drink, more thirst the people will have, consuming more. A can of soda contains an average 150 cal, multiplied by 365 days (1 year) and divided by 3500 cal equivalent to about 500 g of weight, because for every 3,500 eaten and unburned calories, you gain about 500 g of weight [34]. Thereby, is noteworthy that over time the size of can of soda increased, thus there was an increasing in amount of calories, linked with weight gain of according to the Table 2. Interestingly, controlled trial done in schools with obese children found that the obesity decreases when the withdrew soda vending machine. In contrast, the obesity continues the increased in the schools with the soda vending machine [35].

Xenoestrogens are a sub-category of the endocrine disruptor group that imitates the actions of estrogens such Polychlorinated biphenyls

Neolithic period	Worldwide
Organic foods	Processed foods
Good sleep quality	Poor sleep quality
High sunlight's (more vitamin D)	Low Sunlight's (less vitamin D)
Low sanitation	High sanitation
High physical activity	Low physical activity
Higher breastfeeding	Lower breastfeeding
Normal Birth	Cesarean
Without artificial lights and telephone	With artificial lights and telephone
Active lifestyle	Sedentary lifestyle
High intake of fruits and vegetables	Low intake of fruits and vegetables
Absence of fast food consumption	Increase of fast food consumption
Low sugar intake	High sugar intake
Little consumption of antibiotics	Indiscriminate use of antibiotics
Low sodium diet	High sodium diet
Soil rich in minerals (potassium and magnesium)	Soil poor in minerals
Low consumption of alcoholic beverage	High consumption of alcoholic beverage
Low consumption cigarette smoking	High consumption cigarette smoking
Low gluten diet	High gluten diet
Absence dairy, sausages and juices	High dairy consumption, sausages and juices
High water intake	Low water intake

Table 1: Differences of food between Neolithic period and worldwide.

Year/Amount	Calories	Days	Calor X Days	Overal/3500 Cal	Weight/Year
1915...192 mL	82,28	365	30032	8,58	4,29
1955...300 mL	128	365	46720	13,35	6,67
1960...350 mL	150	365	54750	15,64	7,82
1990...590 mL	252,85	365	92290	26,37	13,18
1991...1000 mL	428,57	365	156428	44,69	22,35
1992...2000 mL	857,14	365	312856	89,39	44,69

Table 2: Amount of soda and weight gain.

(PCBs), bisphenol A (BPA), phthalates and are widely used industrial compounds producing adverse effects in human body. Bisphenol A is food contaminant utilized in fabrication of plastics, internal coating cans and it is found in bottles of plastic, plastic containers to store food, drinks and canned foods and children's glass. However, human exposure to BPA could favor obesity [36], metabolic disorders such as hepatic steatosis [37], insulin resistance in childhood obesity [38], and autism spectrum disorder [39]. Recently, phthalates were associated with birth size and gestational that varied by sex and timing of exposure [40], and yet may affect serum thyroid hormones activity in newborns in period neurodevelopment [41].

Heavy metals chemical are elements constituents of the earth crust, however, high levels these compounds can lead the bioaccumulation in the human body and poisoning. These toxins can be introduced into the human body through beverages, foods, skin exposure and the inhaled air it is impossible to live in an environment free of heavy metals. That way, they change the function in glands and organs as enzymes, liver, bone, kidney, brain and heart. Lead can inactive the action of calcium, zinc, iron and when added to arsenic, cadmus and mercury produce synergism, increasing your toxicity. Besides that, they displace the vital nutritional minerals from their original place changing your biological function. On the other hand, as trace elements some metals as zinc, iron, cobalt, copper, selenium, and manganese are essentials for human body [42].

Lard or butter pig is composed of triglycerides obtained from any part of the pig where there is a high proportion of adipose tissue and was used in the past for people to cook. However, she was replaced by vegetables shortening (Crisco) from of 1911, with the strong advertising of be better than butter for you cooking and healthier. Vegetables oils are formed through an industrial process that adds hydrogen to vegetable shortening (partially hydrogenated), that it is cheaper to produce and required no refrigeration. So, the oil is solid at room temperature but melts at cooking temperature increasing shelf life of foods, thereby, there was the costs reduction and in a time when refrigerators were rare [43,44]. Interestingly, after of introduction vegetables oils, the consumption of soybean oil increased more a thousand fold and now supplies about 7% of calories in the U.S diet [45], besides of excess of Omega-6 that is harmful for health. Essential fatty acids as Omega-3 and Omega-6 are crucial for body because he can't produce, must be obtained starting from the diet, but it must get them in a certain balance. In the past, the Omega-6: Omega-3 ratio was in around 4:1-1:2 and currently, the ratio is 16:1 on average with variation between individuals [46]. Added to this, vegetables oils contain large amount Omega-6 that have linked with inflammation [47], cardiovascular diseases [48], diabetes and obesity [49-51], asthma and eczema young children [52], cancer[53,54], homicides rate [55] and depression [56].

Gluten is a compound protein of gliadin and glutenin found in wheat, rye and barley. Gluten is found in biscuits, cakes, pasta, bread, breakfast cereals, flour, pizza bases, soups, sauces and sausages. He helps dough rise providing elasticity, strength and ability to hold food products together while maintaining a palatable texture [57]. In the past, the native diet of humans consisted of meats, fishes, vegetables and fruits with little exposure to grain, however, in the past 500 years the gluten content of foods increased significantly [58,59]. The gluten proteins may have a unique toxicity profile and distinct T-cell stimulatory sequences, as the gliadin, which cannot be degraded by intestinal enzymes and trigger an immune reaction in predisposed individuals. Therefore, the consumption elevated of gluten in modern diet have been linked the gluten-related-disordens as celiac disease and non-celiac gluten sensibility [60], wheat allergy [61], dermatitis herpetiformis [62], gluten ataxia [63], autism [64] and helicobacter pylori infection [65].

Malnutrition

Malnutrition occurs when there a reduction of basic nutrients for human body as the water, vitamins, minerals, amino acids and fatty acids. Nowadays, our food is poor in vitamins and minerals because the famers plan only one type of vegetal or fruit in soil (monoculture) and fertilize the land with little nutrients, thus occur a soil nutrients depletion causing a deficiency these nutrients for the fruits or vegetables. The reduction these nutrients in diet represents the largest public health concern contributor for diseases development affecting quality of life of people in developing and developed countries [66]. Currently, more 805 million people worldwide are undernourished and so, the malnutrition leads to deficiencies of micronutrients as the iron, vitamin A, vitamin B12, folate and riboflavin that are linked the anaemia [67].

Some diseases have been associated with reduction of vitamins, minerals, amino acids and fatty acids. Vitamin D deficiency in general population is associated with increased cardiovascular risk [68], allergic diseases [69], death in the intensive care unit [70], musculoskeletal and autoimmune diseases [71,72] type 2 diabetes [73], obesity and cancer [74,75]. On the other hand, a systematic review found that supplementation with vitamin D_3 decreased cancer mortality by 12% and vitamin D decreased all-cause mortality by 7% among adults [76].

Magnesium is the most common element in the crust of earth and is essential for human health, because it is involved in the homeostasis, protein synthesis and DNA stability [77]. However, there a deficiency of magnesium in general population in worldwide, that contributing for a number of chronic diseases such the migraine [78], epilepsy [79], depression [80], Alzheimer's [81], cardiovascular diseases [82], hypertension [83] type 2 diabetes and insulin resistance [84,85], attention deficit hyperactivity syndrome [86-87] asthma [88] and pre-eclampsia [89].

Hydrocloridric acid (HCl) is produced by parietal cells of stomach in response to ingested protein or fat, however, when eats more than required by body and with the aging, acid production begins to decrease. In the body human, stomach pH is 1.5-3.0 and parietal cells need of iodine, zinc, water and thiamine for produce HCl, thereby, when taking H_2 blockers or proton-pump inhibitors there an increase of pH stomach (>4.0) modifying the function of the digestives enzymes and pyloric valve that are pH dependent. Consequently, the low production of HCl decreased digesting food, amino acids, minerals and vitamins principally the vitamin B12 and folic acid, contributing for abnormal growth bacterial, intestinal dysbiose and immune system deficiency. Thus, favor the appearance of diseases like atrophic gastritis [90], gastro esophageal reflux [91], asthma [92] esophagus Barrett

[93], achlorhydria [94] and increased intestinal permeability that can predispose the autoimmune diseases [95,96].

Iodine is an element crucial for all cell and glands of human body especially for the thyroid in production of hormones thyroid and the iodine deficiency is a global health issue, because the amount of iodine in soil is low and intake dietary in adults is <150 µg/day of according World Health Organization [97,98]. Low intake iodine, selenium, zinc and iron have been linked with type 2 hypothyroidism because the deiodinase enzyme no converted T4 to T3 [99,100]. Iodine deficiency in pregnancy have been associated with cretinism [101], impact neuropsychological development in school aged children [102], in adults iodine deficiency is linked with intestinal failure [103] increase cardiovascular disease risk in overweigh people [104], goiter with complications and impaired mental function [105].

Infections

Nowadays, people in worldwide are dying due to the antibiotic-resistant-bacteria (superbugs) and by infection diseases. Dental infections destroy mitochondria's through of thioesters, gliotoxins and mercury and are the most common diseases in the oral and maxillofacial region. However, majority of chronic systemic disease are due to infection in the tonsils or teeth that are symptom-less and very difficult to detect. Oral infections as the periodontitis can cause the course number of systemic diseases such as cardiovascular disease, cerebrovascular disease, bacterial pneumonia, diabetes mellitus, osteoporosis and adverse pregnancy outcome [106-108]. Cells human body become oxidized by toxins that are pro-oxidant causing damage and altering their normal function and in advanced cancer patients, between two and ten dead teeth, always included root canal teeth. Various study found that all root canal teeth with symptomatic apical periodontitis contain anaerobic bacteria and are a significant source of bacteria and fungi in the circulation blood and endodontic treatment can be the cause of anaerobic bacteremia and fungemia and thereby predispose the various chronic diseases [109-112].

Stress

Currently, all living organism are challenged by internal or external adverse effects that change the homeostasis human body, defined like stress. Noteworthy that worldwide the stress in the people increased and no decreased because work, sedentary, food, sleep loss, socioeconomics and ideological factors, contributing by diseases as hypertension [113], diabetes [114], depression [115], some type of cancers [116], autoimmune diseases [117,118] and autism [119].

Sleep deprivation has been liked with various diseases in worldwide because nothing is healthier by human body that a good sleep night. In the past, we ancestral slept much earlier and totally in the dark, thus there was no failure in production of melatonin by pineal gland. In contrast, the current society sleeps with the light on, television on, tablets and cell phone next to bed. So there a failure of pineal gland in release of melatonin because the lighting of these devices stimulates serotonin production, tricking the pineal gland into thinking it's daytime, confusing the wake/sleep cycle.

Electromagnetic Pollution

Electromagnetic pollution or electromagnetic radiation consists of the waves of energy combining electrical and magnetic fields classified in ionizing radiation and non-ionizing radiation based on its capability of ionizing atoms. Electromagnetic energy that flow along a conductor is a quality power poor also known as dirty energy. Dirty energy is ubiquitous and is generated by electronic equipment as computers, plasma television, energy efficient appliances, dimmer switches, cell phones and broadcast antennas. In buildings, the neighbors who share the same transformer produce dirty energy that contribute by symptoms of radio wave sickness as headaches, dizziness, memory loss, depression, anxiety, palpitations, arrhythmias, pain or pressure in the chest, low or high blood pressure and these symptoms are linked with electrical hypersensitivity [120,121]. Electrical hypersensitivity affect 3% of the population and 35% have your symptoms. Increase of diseases as diabetes, multiple sclerosis, attention deficit hyperactivity disorders, fibromyalgia and asthma chronic fatigue may be linked with electrical hypersensitivity, but reasons still are poorly understood [122]. A study done in laboratory found that insulin release and insulin binding capacity to receptors cell were reduced by electromagnetic fields [123,124]. Besides, it is know that blood sugar levels are increased by stress in diabetics that are exposed the electromagnetic energy induced the stress proteins at various frequencies [125,126]. More investigation this area are need for discovery the mechanisms involved and percentage of population affected.

Conclusion

Malfunction of pancreas, adrenals glands and thyroid that are organs unison is gateway by diseases. In the chronic diseases, the patients have type II hypothyroidism, deficiency of vitamins, minerals, fatty acids, low intake of fruits and vegetables, low physical activity and an excess of fat trans and sugar principally because industrialized food that make part diet in population worldwide. Besides, the toxins, heavy metals dental infection and electromagnetic pollution can contribute for complications in chronic diseases, thus all these factors are crucial for epidemics of chronic and Western diseases, which are currently the biggest health problems in the world. However, the health of patients with chronic diseases is not duty of physicians or health professional since each people will also is responsible by your health.

On the other hand, for help chronic diseases patients, we encourage the physicians and health professional look for these factors in your patients because a disease no appear alone and can be starting point for others. Diseases no remains in healthy body, thereby we suggested that the chronic patients substitute the soft drinks by water with pH alkaline, coconut water and pink salt Himalayan, as these nutrients are crucial for human body. Replace processed food rich in fat Trans by foods made by nature. For removed the pesticides used in plantation, placed foods in one liter of water with 5 mL tincture of iodine 2% by an hour preferably covered and without any contact with light (in the oven) due to the oxidation iodine. Replace vegetables shortening (Crisco) by coconut oil or butter pig for cooking, because excess of Omega-6 is harmful for your health and have been linked inflammation, cardiovascular diseases and diabetes. No have afraid eat fatty, but the good fatty like avocado, linseed, coconut oil and olive oil. Remove or decrease gluten intake can fight and treat gluten-related-disorders, wheat allergy, autism and helicobacter pylori infection.

A seasonal detoxification is crucial for clear your body of toxins speeding up your metabolism for enhance health. Taking one cup of water with one lemon in the morning rehydrates body promotes digestion and help in flow of waste of your body. Protocols of validated detoxification as garlic extract, glutathione, magnesium sulfate and saunas can help in various chronic diseases.

Plantation in monoculture and few fertilizers applied in land were crucial for decrease the vitamins, minerals of grains, fruits and vegetables. Foods are born poor and not replenish all that the body

needs, however, the supplementation of micronutrients, vitamins and minerals offer potential to improve health of patients with deficiency nutritional. Sun exposure between 15-20 min/day at midday (heliotherapy) increase levels vitamin D preventing and treating various diseases, besides of be a way cheap for care of health.

Dental infections have been strongly linked with auto-immune diseases. In doubt, is crucial the physician refer the patient for dentist for check the teeth, fact that is have been common in Germany. Electromagnetic pollution can be reduced through Grahan/Stetzer (GS) filters installed in schools with sick building syndrome. Interestingly, the symptoms of asthma in the students were reduced, student behavior with ADHD improved and students with type 1 and 2 Diabetes control glycemic improved, on the other hand, Grahan/ Stetzer (GS) filters installed in house of patients with multiple sclerosis demonstrated better balance and fewer tremor.

Author Contributors

H.A.P performed literature search, analyzed data, wrote manuscript and had final responsibility for final content. M.C.F.F, L.R.L analyzed data, and assisted with writing the manuscript.

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Effect on Lipid Profile Parameters by the Addition of Orange Juice in Diet of Hypercholesterolemic Patients

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Abstract

Proceedings of epidemiologic data revealed that a more consumption of vegetable and fruits allied with a less susceptibility of Acute Heart Disease. Orange juice is a good source of folate, Vitamin C and flavanones, but their effect on heart patients has not been studied scrupulously. Present research focused on fifty subjects having elevated total cholesterol and LDL cholesterol. Participants consumed 2 cups (5000 mL) of orange juice daily into their diets, each dose over a period of 4 week followed by a 5-week washout period. Plasma lipid, folate and vitamin C (a compliance marker) concentrations measured at baseline, after each treatment, and after the washout period. The consumption of calcium, fatty acids, protein total energy and cholesterol did not significantly varied in each of the period.

The dietary interference had no substantial effect on body weight body mass index or plasma lipid concentration. Though total plasma triglycerol and HDL-cholesterol concentration increased 17% and 24% however LDL-HDL cholesterol ratio declined during the study time period.

Keywords: Lipid; Parameters; Hypercholesterolemic; Orange juice

Introduction

Earlier epidemiologic data revealed that more consumption of vegetable and fruits IS directly proportional to less susceptibility of acute Heart Disease [1]. The advantageous outcome may be allied with inconsequential moieties, particularly flavanones, anticipated to apply their deed by restrain oxidation of LDL and thrombosis [2] and vitamins E, C and carotene which act chiefly as antioxidants [3]. Reduction in homo cysteine was reported through Folic acid found in high concentration in green food stuff and in citrus fruit a transitional component in methionine utilization, which is concerned as a threat for heart disease [4]. Orange and grapefruit juice are good source of folate, Vitamin C and flavanones, but their effect on heart patients has not been studied scrupulously. The foremost element citrus hesperidin, flavonoids naringenin from orange and grapefruits are somewhat analogous to genistein, from soybean thought to be hypocholesterolemic. Thus, juice of fruits containing vitamin E and flavonoids may have positive effect on hyper cholesterolemic patients [5].

Primary flavonoids contents of citrus juice were evaluated for cholesterol breakdown in rodents and HepG2 cell line. In rabbit, 43% and 32% reduction in cholesterol level was noted with orange and grape fruit juice in which experimental cholesterol level was induced by a casein-based and the semi cleaned food with citrus juices (reformed from freeze nectar at 2 times the standard potency. Additionally, esters of liver cholesterol lowered down by 42% but fecal cholesterol elimination or bile acids was not elevated, revealing that juice contents like flavanones, may affect beneficially cholesterol metabolism mainly in the hepatic cells. Parallel to this proposition, decreased level of serum cholesterol was detected due to the sterol *O*-acyltransferase-2 and hydroxylmethylglutaryl-CoA reductase, chief catalyst in metabolism of cholesterol-were repressed in cholesterol-fed mice including blend of primarily citrus flavonoids [6]. Additionally by blocking the synthesis of esters of cholesterol, reduced discharge of polyprotein was observed in incubate HepG2 cells with hesperidin and naringenin simultaneously [7]. Citrus juice contain large amount of flavonoids as well as lemonades which also have apoB-lowering effect in HepG2 [8].

Chances of coronary arteries ailment decreases with the Intake of green food stuff and fruits link and this outcome is accredited primarily to the efficient and preventive activity of phytochemicals and vitamins such as flavonoids and phenols [9]. Diet such as grape, tea and wine supply large amount of flavonoids afterward orange and citrus juice.

Substantial amount of flavonoids and its components are present in Orange juice and its quality depends upon processing conditions of juice. Investigational data revealed that anti-inflammatory, hypolipidemic, anti-carcinogenic and antiallergic characteristics are present in hesperidin and these components aid to minimize the LDL cholesterol concentration in serum [10]. Orange juice along with aerobic exercise helps in lowering the risk of heart and coronary disease by lowering triglyceride and uplifting HDL-cholesterol in obese middle aged women [11]. Vitamin C had vasoprotective effect moreover minor concentration of folate have been linked with reduced concentration of hemocysteine and minimal chances of thromboembolic events.

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Material and Methods

Fifty subjects having elevated total cholesterol and LDL cholesterol included in the study. Participants incorporated 2 cups (5000 mL) of orange juice daily into their diets, each dose over a period of 4 week followed by a 5-week washout period. Plasma lipid, folate and vitamin C (a compliance marker) concentrations were measured at baseline, after each treatment, and after the washout period. At the end of this study the variation in lipid profile parameters by orange juice consumption were observed to evaluate the effect of orange juice on lipid profile in hypercholesterolemic subjects.

First IV blood was collected for baseline testing of lipid profile. Then 2nd IV blood collected after consuming the orange juice for 4 weeks and again 3rd blood sample collected after washout period of five weeks for lipid profile (like Triglycerides, HDL, LDL and VLDL), folate level and vitamin C. Estimation of blood cholesterol level was determined by Chylomicrons, VLDL and LDL were precipitated by adding phosphotungstic acid and magnesium ions to the sample. Centrifugation left only the HDL in the supernatant. Their cholesterol content was determined enzymatically using Cholesterol determination kit.

Statstical Analysis

Obtained results were analyzed by using statistical tool ANOVA (two-way) on Statistical Package for the Social Sciences (SPSS) software version 21.0.0. At the end of this study we were able to conclude the variation in lipid profile parameters by orange juice consumption.

Results

Effect of orange juice consumption on lipid profile

Orange juice and energy intake at initial, after each nutritional level and after the washout period are given in Table 3.1. These data was derived from the diet records of the enrolled subjects. The consumption of calcium, fatty acids, protein total energy and cholesterol were not significantly variant in each of the period. Fiber consumption declined gradually during the study and significantly lowers that initial baseline after the washout period. The propensity for the consumption of dietary decreased during the 1, 2, 3 and washout period may be due to successive increase in orange juice intake through study period.

The dietary interference had no substantial effect on body weight body mass index or plasma lipid concentration. Though total plasma triglycerol and HDL-cholesterol concentration increased 17% and 24% however LDL-HDL cholesterol ratio declined during the study time period. Data shows that pair wise comparison showed that percentage variation in HDL cholesterol and LD-HDL ratio was significantly different in the 3rd from that of 1st and 2nd period the variation in initial plasma concentration observed in 3rd period were significantly associated with the VLDL variation.

At the end of the study period the significantly increased HDL-cholesterol and decreased LDL-HDL cholesterol ratio had not reverted to initial values. In fact, the decrease in the LD-HDL cholesterol ratio and increased HDL-cholesterol at the time of washout tend to be higher as compared to the detected in 3rd period. Moreover it was also observed that subjects consuming processed orange juice showed different results of HDL and LDL-HDL cholesterol concentration, subjects consuming processed orange juice had elevated level of HDL and decreased ratio of LDL-HDL ratio as compared to those who intake fresh orange juice.

Concentration of the HDL cholesterol at the time of wash out and changes at the initial baseline in HDL cholesterol after the wash out were not positively correlated. More-over HDL-concentration did not tend to be elevated in subjects who consumed large amounts of orange juice during the washout period. Plasma triacylglycerols and other indexes [BMI, total VLDL, and LDL cholesterol] which were not influenced by the intake of orange juice were not significantly different from baseline during the washout period.

The results showed, changes in the LDL-HDL cholesterol ratio induced by the intake of 500 mL orange juice/d were significantly inversely related to the initial LDL-HDL cholesterol. Similarly, changes in HDL-cholesterol concentrations tended to be inversely correlated with baseline HDL cholesterol but the association was not significant. Changes in plasma triacylglycerols and plasma folate concentrations induced by the highest dose of orange juice were not significantly correlated with the initial LDL-HDL cholesterol ratio or with the initial HDL cholesterol concentration.

Because many previous studies showed that diet-induced increases in serum folate are associated with decreases in plasma homocysteine concentrations, Concentrations during treatment with the highest dose of orange juice. The results showed no significant relation between the 2 indexes when all data were included. A slight significant inverse correlation was shown after exclusion from the analysis of 2 subjects with unusually high plasma concentrations however, values after the exclusion were still insignificant.

Effect of orange juice consumption on cholesterol level

A sum of 50 hypercholesterolemic subjects was enrolled in this setup in order to analyze the effect of orange juice consumption on cholesterol level with a mean of 250.22 ± 18.5. All of the enrolled patients used to intake 500 ml orange juices daily. Their total cholesterol level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that level of total cholesterol increased successively in each period (257.23 ± 17.9 for 1st, 259.23 ± 17.9 for 2nd and 263.07 ± 17.31) while at the time of washout period level of total cholesterol find to be same as in baseline (Figure 1).

Variables	Initial	1	2	3	Washout
Cholesterol	250.22 ± 18.5	257.23 ± 17.9	259.23 ± 17.9	263.07 ± 17.31	251.02 ± 18.4
Tri-Glycerides	228.26 ± 17.5	228.16 ± 17.5	240.3 ± 17.7	234.5 ± 17.8	262.8 ± 18.3
HDL	41.08 ± 10.08	41.2 ± 10.08	44.6 ± 10.08	47.9 ± 11.3	51.3 ± 12.6
LDL	158.42 ± 13.04	164.2 ± 13.11	159.11 ± 13.21	151.21 ± 13.24	148.22 ± 14.2
VLDL	41.82 ± 9.7	48.06 ± 10.7	41.78 ± 9.7	45.05 ± 9.87	41.91 ± 9.7
Changes in HDL %	----	5	8	19	24
Vitamin C µg/L	9.3 ± 3.4	21.3 ± 4.93	29.3 ± 12.2	37.8 ± 17.3	17.8 ± 7.5
Folate µg/L	35.7 ± 10.2	37.9 ± 12.7	41.6 ± 13.5	45.4 ± 16.7	39.7 ± 10.8

Table 1: Effect of Orange Juice consumption on lipid profile parameter of hypercholesterolemic subjects.

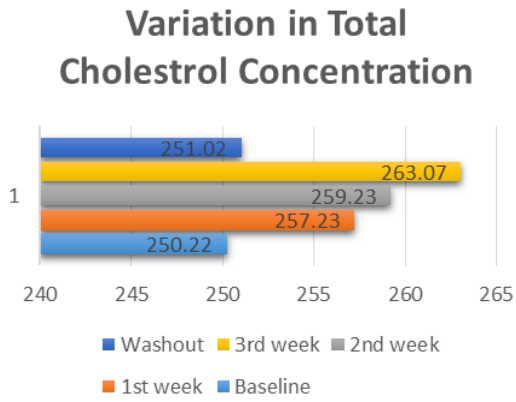

Figure 1: Effect of orange juice consumption on cholesterol level.

Effect of orange juice consumption on tri glyceride concentration level

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on Triglyceride Concentration with a mean of 228.26 ± 17.5. All of the enrolled patients used to consume 500 ml orange juice daily. Their Triglyceride level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that level of Triglyceride concentration increased successively in each period (228.16 ± 17.5 for 1st, 240.3 ± 17.7 for 2nd and 234.5 ± 17.8) it was also observed at the time of washout period level of Triglyceride was elevated (262.08 ±18.3) as compares to initial baseline level (Figure 2).

Effect of orange juice consumption on HDL concentration

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on HDL Concentration with a mean of 41.08 ± 10.08. All of the enrolled patients used to consume 500 ml orange juice daily. Their HDL level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that level of HDL concentration increased successively in each period (41.2 ± 10.08 for 1st, 44.6 ± 10.08 for 2nd and 47.9 ± 11.3) while at the time of washout period level of HDL was elevated (51.3 ± 12.6) as compares to initial baseline level (Figure 3).

Effect of orange juice consumption on LDL Concentration

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on LDL Concentration with a mean of 158.42 ± 13.04. All of the enrolled patients used to consume 500ml orange juice daily. Their LDL level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that level of LDL concentration decreased successively in each period (164.2 ± 13.11 for 1st, 159.11 ± 13.21 for 2nd and 151.21 ± 13.24) while at the time of washout period level of LDL was decreased (148.22 ±14.2) as compares to initial baseline level (Figure 4).

Effect of orange juice consumption on VLDL concentration

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on VLDL Concentration with a mean of 41.82 ± 9.7. All of the enrolled patients used to consume 500ml orange juice daily. Their VLDL level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that level of LDL concentration decreased successively in each period (48.06 ± 10.7 for 1st, 41.78 ± 9.7 for 2nd and 45.05 ± 9.87)

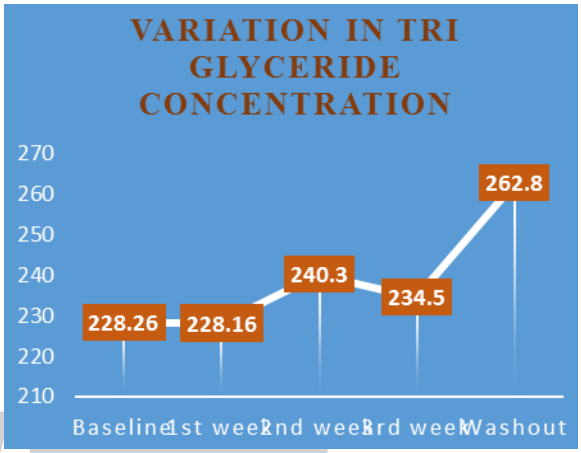

Figure 2: Effect of orange juice consumption on Triglyceride concentration level.

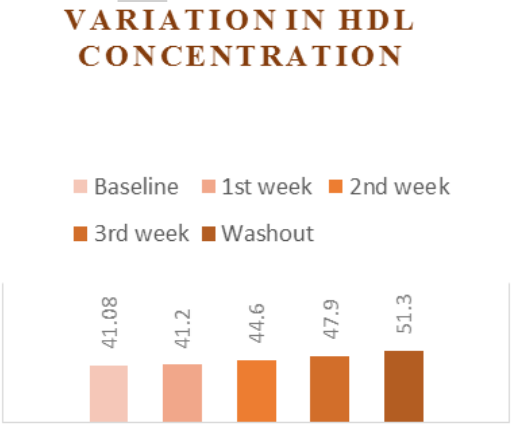

Figure 3: Effect of orange juice consumption on HDL Concentration.

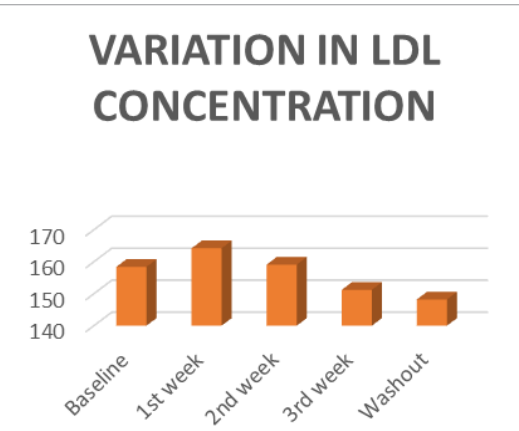

Figure 4: Effect of orange juice consumption on LDL Concentration.

while at the time of washout period level of VLDL was almost same (41.91 ± 9.7) as detected in initial baseline level (Figure 5).

Orange juice: An enhancer of HDL

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on HDL Concentration. All of the enrolled patients used to consume 500ml orange juice daily. Their HDL level was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that HDL concentration increased successively in each period (5% in 1st, 8% for 2nd and 19%) while at the time of washout period level of HDL was significantly elevated (24%) as compared to initial baseline value (Figure 6).

Effect of orange juice consumption on Vitamin C concentration

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration were enrolled in this setup in order to analyze the effect of orange juice consumption on vitamin-C Concentration. All of the enrolled patients used to consume 500 ml orange juice daily. Their vitamin-C level with a mean of 9.3 ± 3.4 was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that vitamin-C concentration increased successively in each period (21.3 ± 4.93 in 1st, 29.3 ± 12.2 for 2nd and 37.8 ± 17.3) while at the time of washout period level of vitamin-C was significantly elevated (17.8 ± 7.5) as compared to initial baseline value (Figure 7).

Effect of orange juice consumption on Folate Concentration

A sum of total 50 subjects with elevated concentration of total plasma cholesterol concentration was enrolled in this setup in order to analyze the effect of orange juice consumption on folate Concentration. All of the enrolled patients used to consume 500 ml orange juice daily. Their folate level with a mean of 35.7± 10.2 was checked after each week and at the time of wash out (Table 1). It was observed from the obtained data that folate concentration increased successively in each period (37.9 ± 12.7 in 1st, 41.6 ± 13.5for 2nd and 45.4 ± 16.7) while at the time of washout period level of folate was significantly elevated (39.7 ± 10.8) as compared to initial baseline value (Figure 8).

Discussion

This study showed, in a group of subjects consisting mainly of individuals with mild-to-moderate hypercholesterolemia, that intake of 500 mL (2 cups) but not of 750 or 250 Ml orange juice/d for 4 week improved the plasma lipoprotein profile by significantly increasing HDL-cholesterol concentrations and by reducing the LDL-HDL cholesterol ratio. The reduction in the LDL-HDL cholesterol ratio observed during treatment with the highest dose of orange juice was entirely due to changes in HDL cholesterol concentrations. This

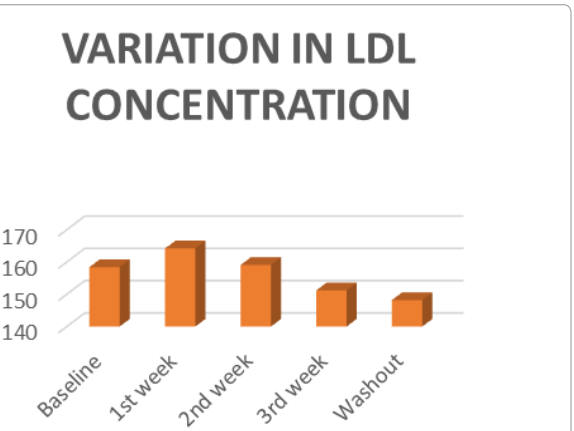

Figure 5: Effect of orange juice consumption on LDL Concentration.

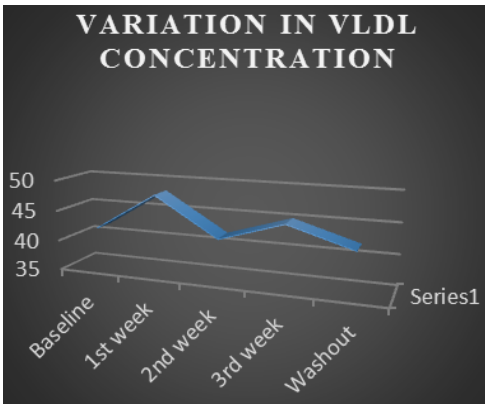

Figure 6: Effect of orange juice consumption on VLDL Concentration.

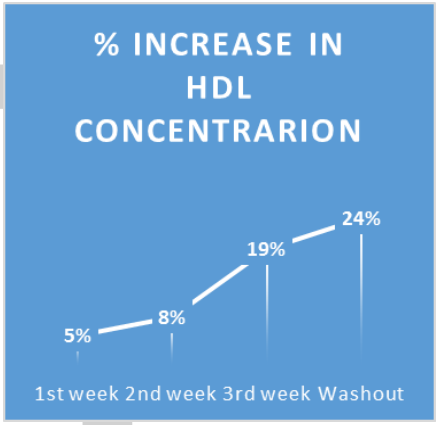

Figure 7: Increase in HDL Concentration.

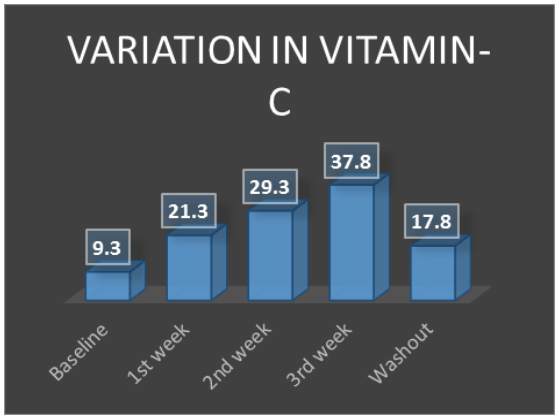

Figure 8: Effect of orange juice consumption on Vitamin C Concentration.

observation contrasts with a substantial reduction in LDL cholesterol induced in orange juice–fed, hypercholesterolemic rabbits [12], with a cholesterol lowering effect of citrus flavonoids observed in rats [6] and with the lack of changes in plasma lipids in normo-cholesterolemic, young men consuming unspecified doses of fresh orange juice for 2 months (Figure 9).

The disagreement between our data in humans and the results in animals could be due to the lower amount of the Juice or its minor components consumed by participants of the study than by rabbits or rats. Differences in responses between Human trials could be due to the fact that some of the participant shad hypercholesterolemia initially and some did not. The observed during the treatment with 250 mL orange juice/d suggest that the beneficial alterations occurred mostly in HDL, a subclass of HDL containing greater proportions of cholesterol but lower proportions of apo A-I than another major HDL subclass, HDL [13]. This response could provide an additional cardio protective effect because previous studies reported a reduction in HDL but no changes in HDL in individuals with coronary heart disease [14].

Significant elevations in plasma triacylglycerol produced in response to treatment with 250 mL orange juice did not exceed the normal range and may not be clinically significant or result in increased cardiovascular risk. Similar changes were also found in hypercholesterolemic rabbits [12] but not in normocholesterolemic men given orange juice. The effect was most likely not due to fructose and sucrose, which are abundant in orange juice, because increases in plasma triacylglycerol induced by these sugars in human trials were associated with a decline rather than an increase in HDL cholesterol [13] and because plasma triacylglycerols were not elevated in rabbits fed grapefruit juice, which consumed only 23% less of both sugars than animals given orange juice [12]. Likewise, previous studies do not suggest that increases in plasma triacylglycerols are due to intake of hesperidin. In rats, serum triacylglycerols were not altered by consumption of a 0.1%-hesperidin diet [6] and actually decreased after consumption of a 10%-hesperidin diet, presumably because of the inhibition of lipase [14]. The 18% increase in plasma folate concentration induced by treatment with the highest dose of orange juice (providing 188.4 g folate/d) was relatively moderate. After similar 4-5 wk periods, more pronounced increases in plasma folate were reported for individuals with coronary heart disease supplemented with 127 µg folic acid/d [15], for healthy subjects who consumed a vegetable- and citrus-rich diet enriched with 350 µg folate/d [16], and for women who consumed 250 µg folic acid/d [7].

However, other studies showed that in young women, a dose of 400 µg folic acid/d increased plasma folate concentrations only after 8 week of consumption and not after 4 week [17]. Treatment with 500 mL orange juice/d did not decrease plasma homocysteine, although its lowest concentrations were generally found in subjects with the highest plasma folate concentrations. The lack of association may have been due to insufficient increases in plasma folate or to a relatively low dose of folate in the juice. A suggested, folate intakes need to be=200 µg/d to produce decreases in plasma homocysteine [18].

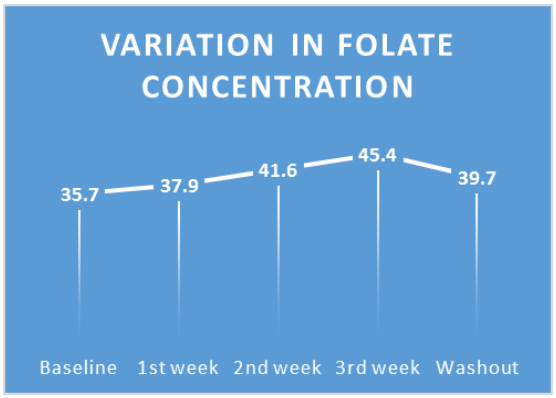

Figure 9: Effect of orange juice consumption on Folate Concentration.

The residual effect of the orange juice intervention on plasma HDL cholesterol and on the LDL-HDL cholesterol ratio observed during the washout period was unlikely due to continued consumption of large amounts of juice by some subjects, as confirmed by a lack of correlation between plasma vitamin C and HDL-cholesterol concentrations during the washout. The tendency toward more pronounced responses observed for plasma HDL cholesterol and for the LDL-HDL cholesterol ratio during the washout period (24% and-17%,respectively) than during period 3 (21% and-16%, respectively) suggests a long-term effect of the juice components, possibly flavonoids, on hepatic lipoprotein metabolism. This could not be verified *in vivo* because after oral administration of orange juice, plasma hesperidin concentrations are below accurate detection limits [19]. However, in our previous study in HepG2 cells, the apo B-lowering effect of citrus flavonoids was partly reversible, implying a long-term effect of these compounds or their metabolites on lipoprotein metabolism in the liver [20].

A significant negative correlation between the baseline LDL HDL cholesterol ratio and the reduction in this ratio caused by the intake of 750 mL orange juice/d indicates that individuals with the highest initial LDL-HDL cholesterol ratio are most likely to experience a reduction in this ratio by consuming orange juice. A similar association was found in hypercholesterolemic subjects after consumption of a diet enriched with soybean products [21]. Our results do not imply that consumption of large quantities of orange juice (20% of daily energy) should be recommended to hypercholesterolemic individuals. According to the US food guide pyramid, a healthy diet includes =5-10 servings of fruit and vegetables daily as well as adequate quantities of fiber. Thus, cardioprotective nutrients in amounts equivalent to those in 500 mL orange juice should be provided from a combination of different foods [21-25].

Conclusion

Epidemiologic data from the present research revealed that a more consumption of vegetable and fruits allied with a less susceptibility of Acute Heart Disease. Orange and grapefruit juice are good source of folate, Vitamin C and flavanones, but their effect on heart patients has not been studied scrupulously. The advantageous outcome may be allied with inconsequential moieties, particularly flavanones, anticipated to apply their deed by restrain oxidation of LDL and thrombosis and vitamins E, C and carotene which act chiefly as antioxidants. The foremost element citrus hesperidin, flavonoids naringenin from orange and grapefruits are somewhat analogous to genistein, from soybean thought to be hypocholesterolemic [26].

Orange juice and energy intake at initial, after each nutritional level and after the washout period derived from the diet records of the enrolled subjects. The consumption of calcium, fatty acids, protein total energy and cholesterol were not significantly variant in each of the period. Fiber consumption declined gradually during the study and was significantly lower than initial baseline after the washout period. The propensity for the consumption of dietary decreased during the 1,

2, 3 and washout period may be due to successive increase in orange juice intake throughout study period.

The dietary interference had no substantial effect on body weight body mass index or plasma lipid concentration. Though total plasma triglyceride and HDL-cholesterol concentration increased 17% and 24% however LDL-HDL cholesterol ratio declined during the study time period. Data shows that pair wise comparison showed that percentage variation in HDL cholesterol and LD-HDL ratio was significantly altered in the 3rd from that of 1st and 2nd period.

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Nutrient Content and Antioxidant Properties of Eggs of the Land Snail *Helix aspersa maxima*

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Abstract

This paper describes selected biochemical and analytical composition of *Helix aspersa maxima* eggs. Analyses determined the percentages of proteins, lipids, sugars, the composition of water and mineral elements, also antioxidant capacity. *H. aspersa maxima* eggs contain the high percentage of protein and are low in lipids (cholesterol content average 0.69 mg%), and are also found rich in Ca. The results showed that the eggs possess antioxidant activity and do not lose the antioxidants during short term storage in salt solution. The results make it possible to evaluate nutritional quality of eggs land snails as a good food source.

Keywords: *Helix aspersa maxima;* Eggs; Antioxidants; Nutrients

Introduction

A *Helix aspersa maxima (Taylor) is* one of the two forms *H. aspersa*, typically antropochorus, widespread through the world in many zones, including temperate and subtropical climates [1]. For commercial breeding, *H. aspersa* is known as *H. aspersa maxima*, which is hermaphrodite snail with determinate grown pattern in many regions, colonizing new habitats [2]. The time for snails to mature and reproduce is 4-7 months [3]. The length of breeding period is between April and October. In some controlled condition [4], like farming system, with control of feed [5], snails can growth in a shade-cloth covered greenhouse giving protection from the sun, wind and predators. So it is possibility to control land snail eggs in March and May (data in present paper). The number of eggs is not significant among the different generation, but reproductive success increased with a number of generations [6]. And therefore *H. aspersa maxima* is the most suitable snail for farming owing it to its adaptability to different environmental conditions, high reproduction and growth rates [7].

The estimated global annual consumption of snail meat is approximately 30 thousand tons, but its fivefold increase is predicted in the next twenty years. The limited opportunities for collecting snails from the natural habitats contributed to the development of helix culture. Increasing number of snail farms in Poland is observed since the late nineties of XX century. Snail farms are being established to produce good quality snails for consumption. One of the most above area of using snail meat and eggs is food industry. Fish roes are consumed for special dinners and invitations all over the world and caviar is a well-known example. The caviar is an expensive product from fish processing industry which is considered as a high-price-flavor with a high nutritional value in international and domestic markets [8]. The rows of sturgeon are known as the original caviar. Land snails eggs are a source in food industry, food processing, also because they are a potential source of healthy food due to its low fat content and presence of nutrients required for a well-balanced diet. The snail meat is mainly consumed as delicacy characterized by excellent nutritious traits and a high dietetic value [9]. However, no studies were published on the nutritional and antioxidant properties of land snail eggs. In that case it is useful to make a basic analyzes for biochemical composition.

Material and Methods

Eggs sample

The sampling of land snail's eggs was performed from snail Farm in Brudnice, Poland. Snails had received the special supplementary for fed 70% rapeseed cake and 30% fodder chalk. Snails were cultivated on peaty soil with 6.5 pH. The samples of eggs were collected as an average laboratory sample from a few thousand snails *H. aspersa maxima.* The samples were collected on the same day of embryonic stage (March–sample 1, 3 and May–sample 2). After sampling, the eggs were refrigerated for 24 h (+4°C), then analyzed. Sample described as 3, was refrigerated in 10% salt solution.

In this study water content (moisture content), reducing sugars, protein, fat, cholesterol content, mineral analysis were determined. Also tests for total antioxidant capacity ($ABTS^{\bullet+}$ reduction and Fe^{3+} reduction) were used.

Water content in eggs

The eggs were washed with deionized water, weighed and dried at 105°C to constant weight, then weighted.

Determination of reducing sugars

Determining the amount of reducing sugars was performed using Somogyi-Nelson assay [10] with a slight modification, using UV-VIS Helios Gamma spectrophotometer (Thermo Spectronic, Great Britain), at the wavelength of 520 nm.

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Protein content

Protein content was determined by the colorimetric method by Bradford [11], using an UV-VIS Helios Gamma spectrophotometer (Thermo Spectronic, Great Britain), at the wavelength of 595 nm.

Cholesterol content

The determination of cholesterol was performed according to Chiocchio and Matković [12]. The samples (2 g of each) were saponificated with 2 g KOH in 25 mL of methanol (HPLC grade). After the saponification (1 h reflux to 70°C), they were left to cool. The supernatant was placed in a funnel and 20 mL of methanol was added twice over the precipitate. The three aliquots in the funnel with 30 mL of dry n-hexane were joined (previously filtered the 60 mL of methanol though Millex-HV 0.45 µm (Millipore S.A., Molsheim, France). This extraction was repeated twice. Fractions were joined and taken to dryness in a Rotavapor (Büchi Labortechnik AG, Switzerland).

The samples were stored (4°C in darkness) until HPLC analysis. Each sample was re-dissolved in 200 µL methanol. For HPLC analysis we used Dionex HPLC system, a flow rate of 1.0 mL/min. The wavelength was set at 290 nm for determinate cholesterol and 254 nm for corticosterone. Thus, each sample was injected twice in the HPLC. The used column was a Hypersil GOLD (5 µm, 250 × 4.6 mm). Methanol: acetonitrile, 80:20 v/v (HPLC-grade) was the mobile phase.

Mineral analysis

The eggs were washed with deionized water, dried at 105°C, weighed and mineralized. The samples were mineralized by treatment with 10 ml of 65% HNO_3 (Suprapur, Merck KGaA, Germany) in two steps working program: heating temperature 200°C, 15 minutes followed by heat at 200°C, 35 min in microwave digestion system (Ethos One, Milestone Srl., Italy). The mineralized sample quantity was about 1 g. After mineralization the samples were diluted with double deionized water (Milli-Q Millipore 18.2 MΩ/cm resistivity). The analysis of selected elements of eggs was performed using ContrAA700 Atomic Absorption Spectrometer (Analityk Jena AG, Germany) with air/acetylene burner. Standards solution were prepared from 1000 mg/L stock solution (CertiPUR, Merck KGaA, Germany) of Fe, Mg, Ca, Zn, Mn, Cr and Cu by dilution with 0,5% HNO_3 (Suprapur, Merck KGaA, Germany). Determination of the investigated components was carried out in three replicates, average results (±SD) being calculated per g dry weight.

Total antioxidant capacity

Antioxidant properties of the snail eggs were assayed with a spectrophotometric method with the use of $ABTS^+$ (synthetic cationradicals) test and the results were presented as Trolox [13] (standard; µmol of Trolox per 1 g of fresh eggs). The absorbance was recorded at 734 nm (UV-VIS Helios Gamma spectrophotometer, Thermo Scientific, Great Britain)

FRAP assay (Ferric ion reducing antioxidant power) was performed according to the methods of Benzie and Strain [14]. Trolox was used as a standard. The absorbance of the samples was determined against blank at 593 nm. The values obtained were expressed as µmol of Trolox per gram of fresh sample.

Statistics analysis

The results were expressed as means +/- standard deviation (SD).

Results and Discussion

Heliculture is an alternative culture in many countries. Also in Poland, the area of snail farm increases. Although the production of good quality snails is favorable a little is known about nutritional exigency [10]. And still the research on snail cultures *Helix aspersa maxima* is rare, despite that these are species with high economic value and a luxury food. The aim of this paper was to present basic analytical and biochemical analyses of the land snail eggs. Some details about eggs, data collection, the storage conditions, eggs weight and moisture content are summarized in Table 1. Wet mass of eggs was between 5.45-6.2 g and dry almost seven times less than wet mass. The moisture content was significant in samples collected in different months (March and May), but in the same embryonic stage (sample 1, sample 2). The percentage of water content in sample storage in 10% salt solution in +4C is lower than in samples storage in +4°C without salt. These results are very similar to studies for snails *H. pomatia* and *H. aspersa* presented by Fontanillas [15] and Bonnet et al. [16].

Rapeseed cake is a high-energy and high-protein feed. It is consisting of heavy-pressed rapeseed and is suitable as a food source for many animals. Total protein content in snail eggs of *H. aspersa maxima* was determined (Figure 1). The content of protein in dry mass was from 34.6% (sample 3) to 42.2% (sample 1), what confirms high content of protein and the fact, that the snails were fed with concentrated high-energy and high-protein rapeseed cake. Researchers confirm that the snail meat contains very high level and quality protein [17]. The data confirm that the protein content found in *H. aspersa maxima* meat was 12% in fresh mass [18], 16,3% in fresh mass [12] and 14.8–18.4% in dry mass [10]. In our study the high content of eggs protein was noticeable. The dry mass of *H. aspersa* contains 10% lipids [19], including phospholipids and cholesterol. Cholesterol in animals is the predominant sterol and in slugs and snails are almost 90% of

Number of sample	Day of collection	Wet weight [g]	Dry weight [g]	Water content [%]
1.	March Sample storage in +4°C	6.1909	0.92361	85.1
2.	May Sample storage in +4°C	5.4511	0.7712	85.6
3.	March Sample storage in 10% salt solution, in +4°C	5.6313	0.8759	84.4

Table 1: Collection of eggs from land snail.

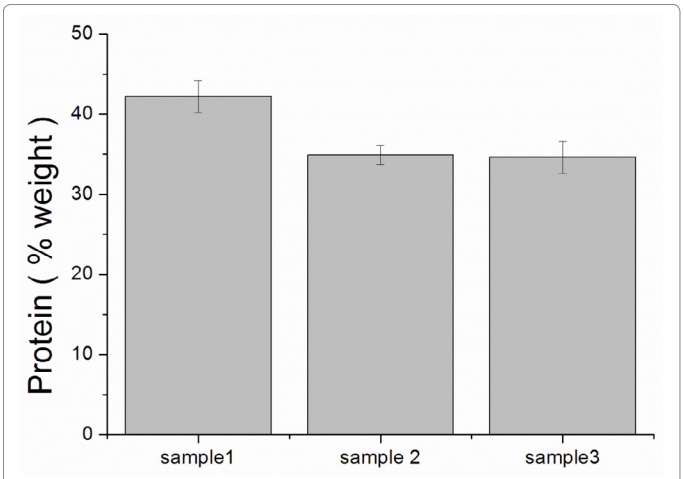

Figure 1: Protein of *Helix aspersa maxima* eggs. Vertical bars represent standard deviations. Sample 1: Eggs collected in March and stored in +4°C; Sample 2: Eggs collected in May and stored in +4°C; Sample 3: Eggs collected in March and stored in 10% salt solution in +4°C.

total lipids [20,21]. In our results average cholesterol content is 0.0077 mg/g. It confirms that cholesterol content in eggs of land snail (0.56 mg%-sample 1) is relatively low comparing to the literature data [22]. In the case of sample 2 and 3 similar results were obtained (Figure 2), so there is no significant difference between time of eggs deposition and preparation of samples (May) and adding eggs to the 10% salt solution (0.77 mg% and 0.75 mg%, respectively). The content of cholesterol was higher for the sample obtained in May (sample 2) and March (sample 3) than for eggs obtained in March and stored only in +4°C. Moreover in human nutrition dietary lipids are indicated as important, also health professionals recommend low cholesterol, low energy and low SFA (saturated fatty acid) diets [10]. According to Murphy [5] snail meat contains little fat. *H. aspersa* eggs in this study do not contain glucose.

Snails are prepared for human consumption in different ways. In *H. aspersa maxima* the visceral hump and foot is removed, head and mantle edge are eaten. Some example of mineral and trace elements from eggs are presented in Table 2. Claeys and Demeyer [18] point richness of *H. aspersa* in mineral salts, nutritive value of relatively low bodyweight. So far there is no data about nutritional value and mineral concentration of snail eggs. But it is known that mineral element in food is important for human health [23]. The potential contribution of 100 g of *H. aspersa eggs* to the Recommended Dietary Allowances (RDA) (US Department of Agriculture, 2004) is presented in Table 3. Likewise to our study, calcium was determined the highest in *H. aspersa* [24,25].

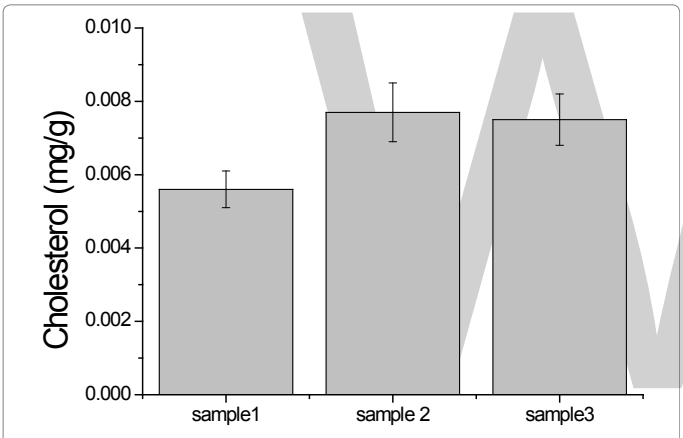

Figure 2: Cholesterol content (mg/g) of dry mass. Sample 1: Eggs collected in March and stored in +4°C; Sample 2: Eggs collected in May and stored in +4°C; Sample 3: Eggs collected in March and stored in 10% salt solution in +4°C.

Species	Part of body	Minerals (mg.100 g^{-1})/DM			Minerals (mg.kg^{-1})/DM			References
		Ca	Mg	Mn	Fe	Cu	Zn	
Helix aspersa maxima	Foot	1390	575	-	112	164	64	[15]
	Viscera	1370	700	-	392	48	1660	[15]
Helix aspersa aspersa	Foot	1620	425	-	84	252	80	[15]
	Viscera	1120	625	-	220	60	1580	[15]
	Meat	-	-	-	5.21	-	-	[20]
Helix lucorum	Foot	1840	400	-	60	228	88	[15]
	Viscera	1040	375	-	720	68	1420	[15]
Helix pomatia	Foot	4580	375	-	144	300	88	[15]
	Viscera	1340	550	-	312	44	2480	[15]
	Meat	-	-	-	17.1	-	-	[21]

DM: Results expressed as % in dry mass; #: Data from analysis described in this paper

Table 2: Mineral content of selected species of snails.

In our research, in breeding snails has been used a fodder chalk. Fodder chalk is a source of concentrated calcium (Ca) and it is used for producing fodder mixtures or for direct feeding in mineral mixtures. This chalk has been used for years in feeding animals as a supplement offering necessary minerals, so indispensable for good health of breeding stock. In our research Ca content was 88.4-117 mg/g dry mass and % of RDA from 107 to 239 in eggs stored in salt solution and refrigerate, respectively. Also the data summarized in Tables 3 and 4 shows that land snail eggs are good source of Ca, Cu and Fe.

Current study presents new data, on the antioxidant capacity of *H. aspersa* eggs using a novel and common methods like ABTS $^{\bullet+}$ test and FRAP assay [26]. The results presented in Table 5 indicate that eggs have high antioxidant capacity. Also the data show no loss of antioxidants during short term storage in salt solution, but comparing the two methods, it is obvious that ferreric reducing assay indicate the higher antioxidant power of eggs. Comparing to the plant extracts the eggs have almost ten times less antioxidant capacity [27].

Element	RDA (mg)	% of RDA supplied by 100 g eggs		
		Sample 1	Sample 2	Sample 3
Fe	10	3.04	5.11	2.57
Mg	420	1.60	2.08	1.23
Ca	1000	239	165	107
Zn	11	2.10	2.41	1.50
Mn	2.3	2.59	3.52	2.88
Cu	0.9	43.01	48.28	35.74

Sample 1: Eggs collected in March and stored in +4°C;
Sample 2: Eggs collected in May and stored in +4°C;
Sample 3: Eggs collected in March and stored in 10% salt solution in +4°C.

Table 3: Contribution of 100 g of *Helix aspersa eggs* and the Recommended Dietary Allowances (RDA).

Element	Nutrient mineral (mg/g dry weigh)		
	Sample 1	Sample 2	Sample 3
Fe	0.0201	0.0365	0.0165
Mg	0.455	0.625	0.325
Ca	88.4	117.4	105.5
Zn	0.017	0.019	0.011
Mn	0.0043	0.0058	0.005
Cu	0.0268	0.0311	0.0223
Cr	0.0014	0.0014	0.0016

Sample 1: Eggs collected in March and stored in +4°C;
Sample 2: Eggs collected in May and stored in + 4°C;
Sample 3: Eggs collected in March and stored in 10% salt solution in + 4°C.

Table 4: Mineral element composition of eggs.

Methods for antioxidant capacity	Sample 1	Sample 3
FRAP assay	2.98 ± 0.09	2.99 ± 0.36
ABTS test	1.17 ± 0.15	0.89 ± 0.25

Sample 1: Eggs collected in March and stored in + 4°C;
Sample 3: Eggs collected in March and stored in 10 % salt solution in + 4°C.

Table 5: Antioxidant capacity of snail eggs.

The results presented by Cagiltay et al. [24] showed that *H. aspersa* is a good source of essential amino acid, fatty acid, vitamin and minerals. Noteworthy, in the nutritional composition of snail are high in protein quality, but low in fat contents, which also coincides with the data of the investigations. In that case snail eggs are an alternative food for people with low fat diet requirements [24,28,29].

Conclusion

Many snail local farms are being established to produce good-quality snail for consumption. Researchers suggested using meat of *Helix aspersa maxima* as a source of food ready for consumption and recommended snails as advantageous foodstuff from a dietary point of view and as a good source of proteins energy value and low in calories [19].

The chemical composition of samples of *H. aspersa* eggs was investigated to determine their nutrition value. The results show that the eggs are high in the water (84.4-85.6% of total) and low in cholesterol (0.77 mg% of total), do not contain glucose. The protein content is high, above 40% of dry mass and is noticeable. Moreover, the eggs are also found rich in Ca and Cu and Fe. The FRAP assay show antioxidant capacity of raw material. Salt solution is a good source to store snail eggs. The results indicate that the *H. aspersa* eggs could be taken as an alternative food source.

Acknowledgements

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Author Contributions

A.G.: Conceived and designed the experiments, performed the experiments, analyzed the data. MD wrote the paper, analyzed the data. BO performed the experiments.

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Luteolin Suppresses Ultraviolet A- and B-induced Matrix Metalloproteinase 1- and 9 Expression in Human Dermal Fibroblast Cells

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Abstract

Ultraviolet (UV) irradiation induces significant changes to skin connective tissues as a result of the degradation of collagen, which is a major structural component of the extracellular matrix. This process may be mediated by matrix metalloproteinases (MMPs). In this study, we examined the protective effect of a polyphenolic flavone, luteolin, on the expression of two matrix metalloproteinases, MMP-1 and MMP-9, in UVA- and UVB-irradiated human dermal fibroblast cells. Luteolin is found in many medicinal plants as well as in a large number of vegetables, fruits and a variety of spices. It has a number of biological activities including anti-cancer, anti-oxidant, anti-inflammatory, anti-allergic and immunomodulatory activities. Human dermal fibroblast (HDF) cells were treated with luteolin at 1-10 μM, then irradiated with UVA at 10 J/cm^2 and UVB at 200 mJ/cm^2. Cells and culture supernatant were harvested 24 h after irradiation.

Our results show that luteolin at 1-10 μM dose-dependently suppressed the expression of MMP-1 and MMP-9 genes in UVA and UVB-exposed HDF cells, as measured by quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR). Luteolin was also found to reduce the production of MMP-1 protein in UVA and UVB-exposed HDF cells detected by enzyme-linked immunosorbent assay (ELISA) in a dose-dependent manner. The release of MMP-9 was also reduced by luteolin in UVB-irradiated HDF cells in a dose-dependent manner.

Our results indicate that luteolin can inhibit UV-induced MMP-1 and MMP-9 expression in HDF cells. Therefore, they may be potentially useful in the prevention and treatment of skin photoaging.

Keywords: Luteolin; Ultraviolet A; Ultraviolet B; Matrix metalloproteinase-1; Matrix metalloproteinase-9; Human dermal fibroblast cells

Introduction

Ultraviolet irradiation causes distinct changes to skin collagenous tissues as a result of the breakdown of collagen, a major component of the extracellular matrix. UVA (long length, 320-400 nm) and UVB (short length, 290-320 nm) tend to be associated with oxidative processes involved in photo aging. Exposure of the skin to UVA and UVB induces the intracellular generation of large quantities of reactive oxygen species (ROS). ROS-induced molecular damage produces a number of harmful effects on cellular function and homeostasis, while degradation of extracellular matrix (ECM) proteins, such as collagen, by ROS can cause major changes in skin connective tissue [1-5]. A cascade of gene expression is initiated following UV-irradiation, which results in the upregulation of MMPs such as MMP-1 (collagenase) and MMP-9 (gelatinase B). These alterations in the ECM, mediated by MMPs, are known to be a cause of skin wrinkling that characterizes premature skin aging and aged skin [1]. MMPs are zinc-dependent endopeptidases. To date, more than 20 members of the human MMP family have been described, but depending on their structure and substrate specificity, they are divided into subgroups: collagenases, stromelysins, stromelysins-like MMPs, gelatinases, membrane-types MMPs (MT-MMPs), and other MMPs [6]. MMPs are produced by many different types of cells including keratinocytes, fibroblasts, endothelial cells, monocytes, lymphocytes and macrophages [7]. MMP-1 and MMP-9 are capable of degrading collagen, which is the main structural protein (ECM) found in connective tissue in animals. Recently, it was suggested that excessive matrix degradation by UV-induced MMP-1 and MMP-9 secreted by keratinocytes and fibroblast cells contributes substantially to connective tissue damage that occurs during photo aging [8,9]. This evidence suggests that the expression of MMP-1 as well as MMP-9 plays a major role in the process of photoaging in fibroblast cells.

Luteolin, 3′,4′,5,7-tetrahydroxyflavone, is a flavone that is found in many medicinal plants, a large number of vegetables, fruits and a variety of spices (Figure 1). Dietary sources of luteolin are celery, green pepper, thyme, perilla, parsley, sage, carrots, and oregano [10]. Luteolin has many biological activities including anti-cancer, anti-oxidant, anti-inflammatory, anti-allergic and immunological activities [11-18]. Although luteolin has been tested in various bioactivity assays, only few studies have dealt with its modulation on gene expression and MMP protein production during the photo aging of human fibroblast cells. In the present study, we assessed the effect of luteolin on matrix metalloproteinase MMP-1 and MMP-9 activities in UVA- and UVB-irradiated human dermal fibroblast cells.

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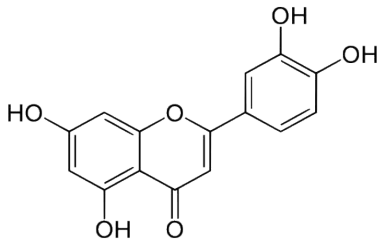

Figure 1: Chemical structure of luteolin.

Materials and Methods

Cell culture and UV irradiation

Normal human dermal fibroblast cells were purchased from (Lonza, Walkersville, MD, USA) and cultured in growth medium FGM™-2 BulletKit™ (Lonza) supplemented with 500 mL FBM® (fibroblast basal medium), 10 mL FBS (fetal bovine serum), 0.5 mL GA-1000 (gentamicin sulfate amphotericin-B), 0.5 mL insulin (recombinant human) and 0.5 mL rhFGF-B (r-human fibroblast growth factor-B) at 37°C in a humidified atmosphere containing 5% CO_2. HDF cells were maintained until 70-80% confluence then 1×10^5 cells /well/500 µL on a 24-well plate were pretreated with luteolin (Wako Pure Chemical Industries, Ltd. Osaka, Japan) at concentration ranging from 1 to 10 µM in 0.1% (v/v) dimethyl sulfoxide, DMSO (Wako Pure Chemical Industries, Ltd.) or DMSO alone (for control) in complete growth medium for 24 h. Medium was removed and cells were washed twice by phosphate-buffered saline, PBS (Takara Bio Inc., Shiga, Japan). Hank's balanced salt solution, HBSS (Sigma-Aldrich, Co., St. Louis, USA) was added and the cells were irradiated with UVA at (5, 10 J/cm^2) and UVB at (100, 200 mJ/cm^2) using a UV irradiator (NS-8F; Sanwa Medical, Saitama, Japan). The intensity of irradiation was monitored with the use of a UV intensity meter (model ATV-3W, product code: 3534032, Atto Corporation, Tokyo, Japan) and a photodetector for UVB (model CX-312, product code: 3534036, Atto Corporation,) and for UVA (model CX-365, product code: 3534037, Atto Corporation) with a UVB cutoff filter (Schott WG 345; UQG Ltd., Cambridge, England), positioned at the same distance from the UV source as the cells. During irradiation, control cells were treated identically, but without the exposure to UV light. After irradiation, HBSS was removed; fresh complete growth medium was added in the presence of the indicated concentration of luteolin and incubated for 24 h. Each concentration was tested in duplicate (n=2). In addition, we have determined the expression levels of MMP-1 and MMP-9 with the indicated doses of UV in a different time period (3 h, 6 h, and 24 h). We found that UVA (10 J/cm^2) and UVB (200 mJ/cm^2) for 24 h was the best for MMP-1 and 9 gene expression (data not shown). Therefore, UVA at (10 J/cm^2) and UVB at (200 mJ/cm^2) were used throughout the experiments.

Cell viability

To confirm the effect of UV on HDF cell viability, 1×10^5 cells/well/500 µl on a 24-well plate were exposed to UVA (10 J/cm^2) and UVB (200 mJ/cm^2) irradiation in the present of Hank's balanced salt solution, HBSS (Sigma-Aldrich). After exposure to UV, complete growth medium was replaced and cells were incubated for 24 h. Total cell viability was assessed by the alamarBlue® assay in which cells were treated with alamarBlue® (Bio-Rad Laboratories, Inc. Trek Diagnostic Systems, USA) for 3 h. Absorbance was measured at 570 nm and 600 nm with a microplate reader (SpectraMax® 190, Molecular Devices corporation, Sunnyvale, CA, USA) and results were analyzed by a software (Soft max Pro5.2, Molecular Devices corporation).

Enzyme-linked immunosorbent assay (ELISA)

To investigate the effect of luteolin on the release of MMP-1 and MMP-9 on UV-irradiated HDF cells, cell culture medium was measured by ELISA. Semi-confluent (70-80%) HDF cells were seeded into 24-well plates and pretreated with luteolin at 1, 2, 5 and 10 µM and incubated for 24 h. On the next day, the supernatant was removed, cells were washed with PBS, HBSS was added and then cells were irradiated with UV, fresh medium was replaced, test samples were added once more and incubated for 24 h. ELISA was performed with the culture medium supernatant according to the manufacturer's instructions (human total MMP-1, MMP-9, R&D Systems®, Minneapolis, MN, USA). Absorbance was measured at 450 nm by a SpectraMax® 190 microplate reader and the result was analyzed by Soft max Pro5.2 software.

Quantitative real-time reverse transcription–polymerase chain reaction (qRT-PCR)

Total RNA was extracted from cultured cells using an RNA extraction kit (NucleoSpin® RNA II Takara, MACHEREY-NAGEL GmbH & Co. KG., Düren, Germany) and quantified spectrophotometrically. Approximately 2 µg of RNA was used as a template for the synthesis of cDNA using the PrimeScript™ RT reagent kit, Perfect Real Time (Takara Bio Inc., Shiga, Japan) according to the manufacturer's protocol. To prevent possible contamination by genomic DNA, samples were treated with deoxyribonuclease (RT-grade; Wako Pure Chemical Industries, Ltd.) as recommended by the manufacturer. qRT-PCR was performed using the Thermal Cycler Dice Real Time (TaKaRa Bio., Tokyo, Japan) according to the manufacturer's instructions. Amplification conditions were as follows: 95°C for 30 s followed by 40 cycles consisting of 95°C-5 s, 60°C-60 s, 1 cycle: 60°C-30 s and 95°C-15 s. Primers were purchased from TaKaRa Bio., and are listed in Table 1.

Statistical analysis

All experiments were repeated at least three times. Data is presented as the mean ± standard error (SE). Statistical analysis was determined by the one-way analysis of variance (ANOVA). A P value of less than 0.05 was considered to be statistically significant.

Results

Effects of UVA and UVB on viability of HDF cells

To observe whether the doses of UV light applied induced toxicity, the viability of HDF cells was measured by the alamarBlue® assay. Human dermal fibroblast (1×10^5 cells/well/500 µL on a 24-well plate) were exposed to UVA (10 J/cm^2) and UVB (200 mJ/cm^2) irradiation and cells were incubated for 24 h. Total cell viability was assessed by the alamarBlue® assay in which cells were treated with alamarBlue® (Bio-Rad Laboratories, Inc. Trek Diagnostic Systems, USA) for 3 h. The cell viability was tended to be reduced but not significantly different between UV-exposed and control HDF cells (data not shown).

Luteolin suppresses UVA- and UVB-stimulated MMP-1 and 9-production in HDF cells

UV-irradiation damages human skin tissues and causes premature skin aging or photoaging by activating MMPs, which are responsible for the degradation of collagen, a major structural component of the extracellular matrix. To examine the effect of luteolin on the production of MMP-1 and MMP-9 in HDF cells, cells were exposed to UVA (10 J/cm^2) and UVB (200 mJ/cm^2) and MMP-1 and MMP-9 was measured in cell culture supernatants by ELISA. The results show that UVA and UVB induced the production of MMP-1 as well as MMP-9 in

HDF cells. The addition of luteolin reduced the production of MMP-1 in UV-irradiated cells in a dose-dependent manner (Figures 2 and 3). The release of MMP-9 was also reduced by luteolin (Figure 4) in UVB-irradiated HDF cells, but there was no change in UVA-irradiated cells (data not shown).

Luteolin suppresses UVA- and UVB-stimulated MMP-1 expression in HDF cells

To further ascertain whether luteolin regulates the expression of MMP-1 at the gene level, expression of the MMP-1 gene was examined in both UV-irradiated HDF cells. Cells were seeded into 24-well plates and pretreated with luteolin at 1-10 μM and incubated for 24 h. and cells were irradiated with UV, test samples were added once more and incubated for 24 h. Total RNA was extracted from cultured cells and MMP-1 expression was quantified by qRT-PCR. When HDF cells were exposed to UV, expression of the MMP-1 gene was up-regulated. The inclusion of luteolin in the culture medium decreased the expression of the MMP-1 gene against both UV-irradiated HDF cells in a dose-dependent manner.

Luteolin suppresses UVA- and UVB-induced MMP-9 expression in HDF cells

MMP-9 plays a prominent role in the breakdown of the extracellular matrix. In this process, collagen is cleaved by collagenase, and cleaved collagen is further degraded into gelatin and small peptides by gelatinase B, and induces breakdown of the ECM, which is a major factor responsible for wrinkle formation [19]. Total RNA was extracted from UV-irradiated HDF cells treated with luteolin and MMP-1 expression was quantified by qRT-PCR. Results demonstrated that UV-irradiation increased MMP-9 expression in HDF cells. The inclusion of luteolin in the culture medium decreased the expression of the MMP-9 gene against both UV-irradiated HDF cells in a dose-dependent manner.

Discussion

Luteolin is a polyphenolic flavone found in a large number of vegetables, fruits and spices (Figure 1) [1]. A number of studies have reported on the beneficial effects of luteolin [11,20-23] but only a few studies have focused on its anti-photoaging effects. Ultraviolet irradiation, both UVA and UVB, penetrates the atmosphere and plays an important role in premature skin aging, skin cancer and suppression of the immune system. Photodamaged skin induces certain MMPs that breakdown dermal matrix protein such as collagen and elastin of the extracellular matrix [3,4,24-25]. It was recently established that fibroblast cells secrete MMP-1 and MMP-9 against UV during photoaging [8,9]. Therefore, the suppression or inhibition of MMP-1 and MMP-9 overexpression induced by UVA and UVB could constitute a novel application to reduce photo aging.

Flavonoids, including epigallocatechin gallate (EGCG) [26], curcumin [27], and resveratrol [28] in the micro molar concentration range inhibit UV-exposed metalloproteinases activity *in vitro* and *in vivo*. However, a few studies have investigated the direct modulatory effects of luteolin on MMPs during photoaging [29-33]. Moreover, to date, a relationship between luteolin and MMP-9 against both UVA and UVB-irradiation has not been demonstrated, despite the importance of MMP-9 and collagen breakdown during photoaging using HDF cells [19]. To the best of our knowledge, this is the first report to show that luteolin suppresses both UVA and UVB-induced MMP-1 and MMP-9 in HDF cells in a dose-dependent manner.

Similar to our results, luteolin at 1-5 μM significantly inhibited UVA (15 J/cm^2)-induced MMP-1 mRNA expression in human keratinocyte HaCaT cells [29]. In another study, luteolin at 5-10 μM inhibited UVB (10 mJ/cm^2)-induced MMP-1 expression in a dose-dependent manner in human keratinocyte HaCaT cells [30]. Human dermal fibroblast cells exposed to UVA (6.3 J/cm^2) induced MMP-1 when luteolin was inoculated into the cell culture at 1-10 μM, reducing the expression of MMP-1 protein and mRNA [31]. Another study by Wölfle et al. showed that luteolin at 4 μg/mL inhibits MMP-1 expression against UVA-1 (20 J/cm^2)-induced human dermal fibroblast cells [32].

We showed the protective effect of luteolin at 1–10 μM (non-cytotoxic levels) [31] against both UVA (10 J/cm^2) and UVB (200 mJ/cm^2)-irradiated HDF cells. Our results demonstrate that luteolin suppresses both UVA and UVB-induced MMP-1 production at protein and gene expression levels (Figures 2-6). We also found that luteolin suppresses both UVA and UVB-induced MMP-9 gene expression

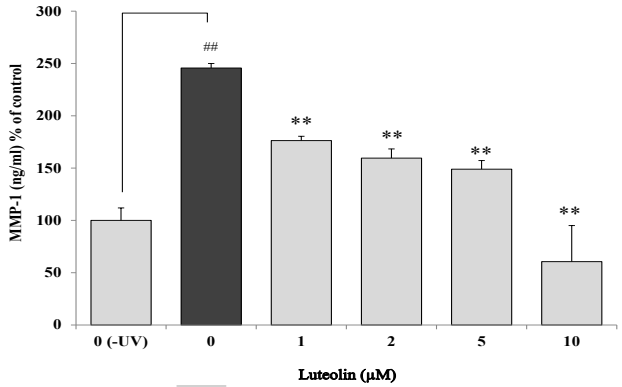

Figure 2: Effects of luteolin on MMP-1 protein release was quantified by enzyme-linked immunosorbent assay (ELISA). HDF cells were treated with different doses of luteolin for 24 h and then the cells were exposed to UVA (10 J/cm^2) irradiation. After exposure to UVA, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. ELISA was performed with the supernatant of the culture medium. Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

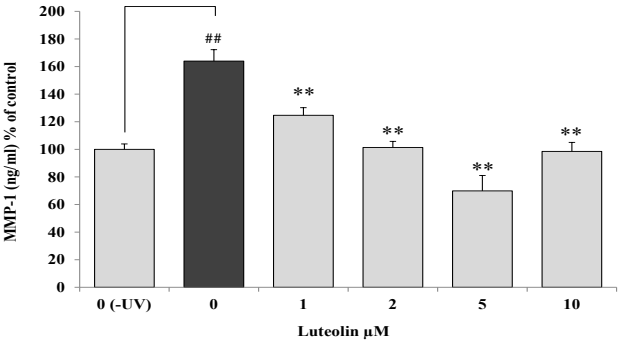

Figure 3: Effects of luteolin on MMP-1 protein release was quantified by enzyme-linked immunosorbent assay (ELISA). HDF cells were treated with different doses of luteolin for 24 h and then the cells were exposed to UVB (200 mJ/cm^2) irradiation. After exposure to UVB, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. ELISA was performed with the supernatant of the culture medium. Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

Gene name	Forward primer	Reverse primer	Sizes (bp)
Human MMP-1	5′-ATTCTACTGATATCGGGGCTTTGA-3′	5′-ATGTCCTTGGGGTATCCGTGTAG-3′	408
Human MMP-9	5′-CTGCCAGGACCGCTTCTACT -3′	5′-TGGTCCCAGTGGGGATTTAC-3′	153
Human ActB	5′-TGGCACCCAGCACAATGAA -3′	5′-CTAAGTCATAGTCCGCCTAGAAGCA-3′	185

Table 1: Primers used for Q-PCR.

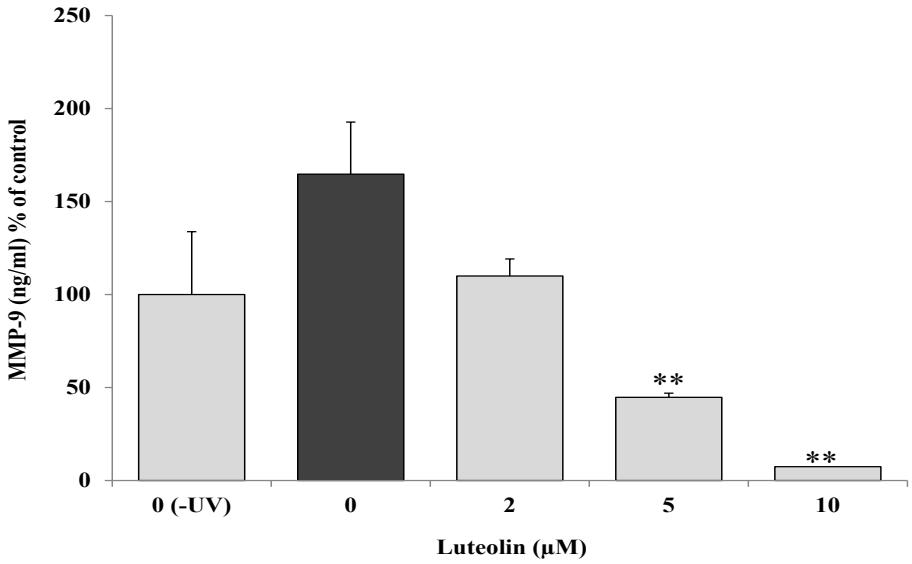

Figure 4: Effects of luteolin on MMP-9 protein release was quantified by enzyme-linked immunosorbent assay (ELISA). HDF cells were treated with different doses of luteolin for 24 h and then the cells were exposed to UVB (200 mJ/cm^2) irradiation. After exposure to UVB, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. ELISA was performed with the supernatant of the culture medium. Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

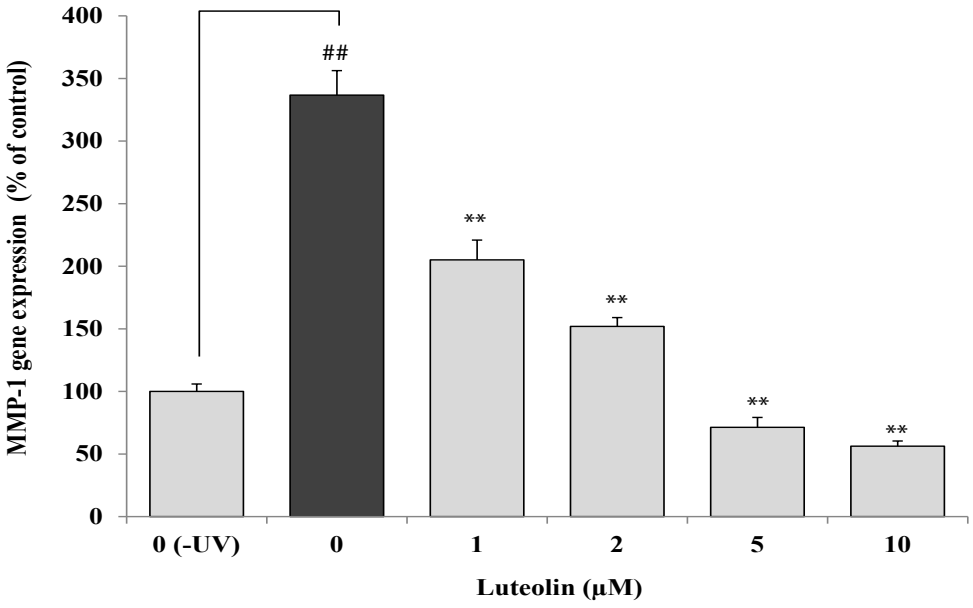

Figure 5: Effects of luteolin on MMP-1 gene expression in UVA-irradiated HDF cells. HDF cells were treated with different doses of luteolin for 24 h and then exposed to UVA (10 J/cm^2) irradiation. After exposure to UV, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. RNA was extracted from the cells, and MMP-1 gene expression was determined by qRT-PCR. Results were normalized using the level of Beta-actin (ActB). Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

(Figures 7 and 8) as well as protein production against UVB-induced HDF cells (Figure 4). However, the level of MMP-9 protein did not change in UVA-irradiated HDF cells. On the other hand, MMP-9 gene expression was much stronger than that of MMP-1. The variance observed in the data pertaining to gene expression and protein release of MMP-9 in HDF cells observed in our experiment might be due to biological differences in abundance between transcript and protein. Further studies are needed to resolve this discrepancy.

UV light irradiation induces signal transduction in skin photo aging. The activation of cell surface receptors, including cytokine receptors and growth factor receptors, by UV irradiation stimulates signal transduction pathways involving MAP kinase family members, including P13K and AKT, ERK, JNK and P38 which lead to activation of the activator protein-1 (AP-1) and nuclear factor kappa B (NF-kappaB) transcription factor family members. This activation results in an increase in the expression of several MMPs, including MMP-1, MMP-3 and MMP-9 [34,35].

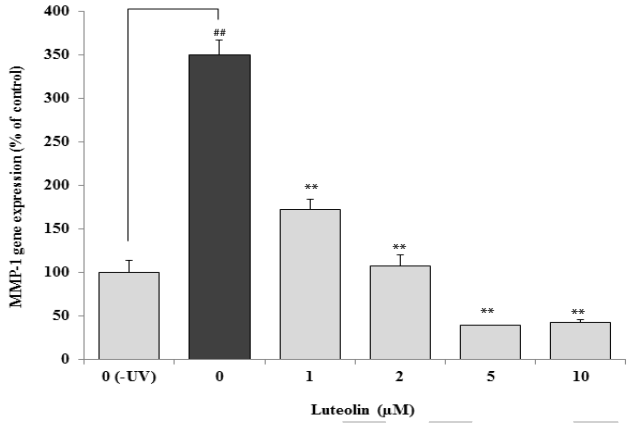

Figure 6: Effects of luteolin on MMP-1 gene expression in UVB-irradiated HDF cells. HDF cells were treated with different doses of luteolin for 24 h and then exposed to UVB (200 mJ/cm^2) irradiation. After exposure to UV, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. RNA was extracted from the cells, and MMP-1 gene expression was determined by qRT-PCR. Results were normalized using the level of Beta-actin (ActB). Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

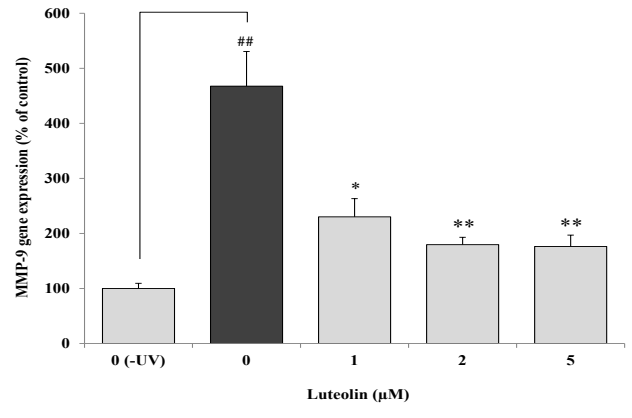

Figure 7: Effects of luteolin on MMP-9 gene expression in UV-irradiated HDF cells. HDF cells were treated with different doses of luteolin for 24 h and then exposed to UVA (10 J/cm^2) irradiation. After exposure to UV, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. RNA was extracted from the cells, and MMP-9 gene expression was determined by qRT-PCR. Results were normalized using the level of Beta-actin (ActB). Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

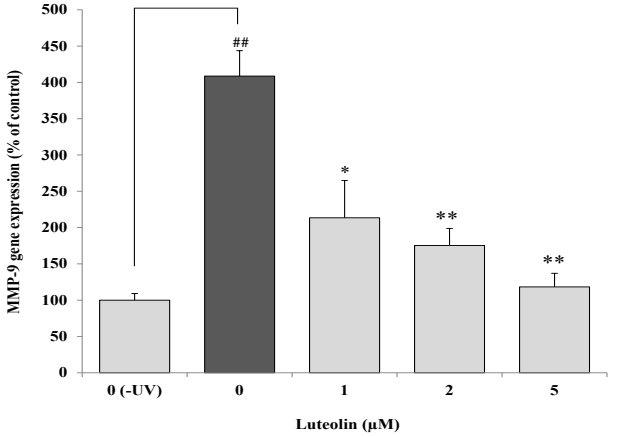

Figure 8: Effects of luteolin on MMP-9 gene expression in UV-irradiated HDF cells. HDF cells were treated with different doses of luteolin for 24 h and then exposed to UVB (200 mJ/cm^2) irradiation. After exposure to UV, fresh complete growth medium was added in the presence of the indicated concentrations of luteolin and incubated for 24 h. RNA was extracted from the cells, and MMP-9 gene expression was determined by qRT-PCR. Results were normalized using the level of Beta-actin (ActB). Data are expressed as a relative percentage value of the control. Results are mean ± SE. *P<0.05, **P<0.001 compared to UV-irradiated samples.

In conclusion, this study demonstrated that both UVA and UVB activate HDF cells and lead to the release of MMP-1 and MMP-9. Furthermore, luteolin at a micro molar concentration range inhibits the production of MMPs at the protein and gene levels. Luteolin may also suppress the development of skin photo aging.

Acknowledgment

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Effect of Transplanting Dates on Cooking, Milling and Eating Quality Parameters of Some Fine and Coarse Grain Rice Lines

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Abstract

In order to elucidate the effects of transplanting date on milling, cooking and eating quality traits of nine coarse grain and ten fine grain *indica* rice lines under four sowing date treatments were studied. Results suggest that very early transplanting is more damaging to milling and cooking of both, fine as well as coarse grain rice lines as compared to delayed transplanting. Likewise, much delayed transplanting is more destructive for milling and cooking characters in case of fine grain rice lines as compared to coarse grain type rice lines. In case of studied fine grain rice genotypes, head rice recovery was observed maximum when translated at 18th of June. Similarly, cooked grain length was improved significantly in case of fine rice lines with delay in transplanting. Results suggest fine grain rice varieties to be transplanted before onset of July in order to have least broken rice in milled rice. Delayed sowing date, milling quality, total and head rice recovery, cooked grain length and bursting percentages showed different trend with respect to the rice lines. KSK 133 and Basmati 515 showed maximum head rice recovery among coarse and fine grain rice lines respectively. Likewise, PK 8785-1-1 and PK 8671-24-4-1-20 showed maximum cooked grain length among coarse and fine grain rice lines respectively.

Keywords: Milling; Cooking; Quality traits; Fine grain; Coarse grain; Rice lines

Introduction

Rice is an important food crop of world and feeds almost half of the world's population. Rice in Pakistan holds an extremely important position in agriculture and the national economy. Pakistan is the world's 11th largest producer of rice, after China, India, Indonesia, Bangladesh, Vietnam, Thailand, Burma, Philippines, Brazil and Japan. Rice is the second largest staple food crop and is also an exportable item. It accounts for 3.2% in the value added in agriculture and 0.7% of GDP. During July-March 2014-15, rice export earned foreign exchange of 1.53 billion USD. During 2014-15, rice was sown on an area of 2891 thousand hectares showing an increase of 3.6% over last year's area of 2789 thousand hectares. Rice recorded highest ever production at 7005 thousand tonnes, showing a growth of 3.0% over corresponding period of last year's production which was 6798 thousand tonnes (Economic Survey of Pakistan 2014-15). Rice is grown in all five provinces of Pakistan, its mainland is plain areas of Province Punjab. Pakistan stands among the leading exporters of rice in the world, and is known for its cooking quality i.e., longer grain length special taste and aroma, which can be produced nowhere else but in "Kallar Track" of Pakistan.

Kallar Track in an area in Punjab which includes District Sialkot, Narowal, Gujaranwala, Hafizabad, Shiekhupura and some part of District Lahore. Due to the presence of heavy clay soil enriched with calcium carbonate the trait of aroma can only be expressed in this soil. Pakistani basmati rice is a source of foreign exchange earning More than 1.36 million tonnes of rice worth 507 million USD were exported in the 2014-15 fiscal year (Ministry of Commerce Pakistan). Being an agrarian based economy, Pakistan's economic growth depends upon progress in agricultural research. In rice sector, there is only one known public sector rice research institution in Pakistan: Rice Research Institute, Kala Shah Kaku. While conducting research on rice many management practices are adopted to check the effect on its quality and production.

Transplanting date is a key factor which affects quality of basmati and coarse grain rice cultivars. To acquire higher paddy yield of better quality, coarse grain varieties may be transplanted from mid-June to early July. Pakistani farmers are demanding superior rice grain quality varieties for different reasons [1-3]. The of rice grain quality parameters includes many components such as appearance, cooking, milling and eating qualities. Among these, consumers often pay more consideration to appearance after cooking [4]. Genetic and environmental factors both confer great effect on rice grain quality, especially photo-periodism and temperature at the heading and doughing stage. There are increase chances of occurrence of chalky grain and reduction of the head rice ratio because of high temperature during the heading stage [5,6]. The optimum temperature to produce superior quality rice is about to be 25°C at the filling stage [7]. The reason for deterioration of rice quality is because of the high temperatures at grain filling and doughing stage adversely affect kernel development and reduce the carbohydrates in the plant, leading to a decrease in the head rice recovery as well as cooking traits [8-10].

The grain dimensions of both paddy and milled rice was affected by sowing and transplanting date. Bran percentage was significantly increased with late transplanting dates, however decrease in amylose content occur. Late transplanting dates affect the cooking time as it decrease the cooking time but increased the solid losses in gruel. Similarly late transplanting deteriorated the organoleptic features of cooked rice and had higher values for clearing and spreading [11]. Different rice varieties showed Significant variation in rice quality

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characters (head rice and brokens) tested under different transplanting dates [12]. For this reason, it is necessary to evaluate the performance and quality characteristics of rice cultivars/promising lines at different transplanting dates in order to measure the effect of high temperature and photo-sensitive during the ripening stage.

Material and Methods

Research study was carried out in Rice Technology section of Rice Research Institute, Kala Shah Kaku where grain shape, appearance, cooking, milling and eating quality traits of rice breeding material comprising of nine coarse grain and ten fine grain *indica* rice lines transplanted under four transplanting date treatments were studied. Physical characteristics include milling recovery (Brown Rice, Total Milled Rice and Head Rice percentages) and cooking quality (Cooked Grain Length and percentage of grains bursting upon cooking).

The objective of this experiment is to ascertain the optimum time (date) of transplanting for obtaining higher milling recovery and best cooking quality in advanced coarse grain rice lines. There were eight advance coarse grain lines and nine fine grain lines including two check varieties from each group of grain type transplanted at four different dates by the Agronomy Section of this institute. Transplanting dates were kept in the main plots while varieties/lines in sub plots. After harvesting from the field, paddy samples were cleaned, dried to 10% moisture content and milled in the Rice Technology Laboratory. The data on milling recovery and cooking quality of these lines were determined and compared with standard check variety of KSK 133 (coarse grain type) and Basmati 515 (fine grain type).

Results and Discussion

The main results showed that the effects sowing date and cultivars on the grain qualities were highly significant (Tables 1 and 2). Delayed sowing date, milling quality, total and head rice recovery, cooked grain length and bursting percentages showed different trend with respect to the rice lines (Figures 1-4). However, as depicted in Figure 1, in case of coarse grain rice lines, changing the translating dates had not significant effects on brown rice % and total milling recovery. However, head rice recovery was affected significantly due to different transplanting dates. As depicted in Figure 1, average HR% of all the studied coarse

	BR (%)	TMR (%)	HR (%)	CGL (mm)	B (%)
D1	78.9	70.3	48.8	11.3	7.6
D2	79.7	71.2	55.4	11.6	4
D3	80.3	71.3	59.4	11.5	3.7
D4	79.9	72	62.8	11.6	4.4
S.D. (Mean)	± 0.589	± 0.698	± 6.016	± 0.141	± 1.806
Average	79.7	71.2	56.6*	11.5	4.925*
D1: 5th May; D2: 27th May; D3: 18th June; D4: 10th July					

Table 1: Averaged values of cooking, milling and eating quality parameters of some coarse grain rice lines as influenced by different transplanting dates.

	BR (%)	TMR (%)	HR (%)	CGL (mm)	B (%)
D1	78.8	68.7	41.3	12.4	15.5
D2	78.8	69.1	46.6	13.8	14.7
D3	79.9	70.6	54.3	13.9	6.1
D4	80	70.7	52.5	14.8	2.6
S.D. (Mean)	± 0.665	± 1.024	± 5.915	± 0.991	± 6.377
Average	79.4	69.8	48.7*	13.7	9.7**
D1 = 5th May; D2 = 27th May; D3 = 18th June; D4 = 10th July					

Table 2: Averaged values of cooking, milling and eating quality parameters of some fine grain rice lines as influenced by different transplanting dates.

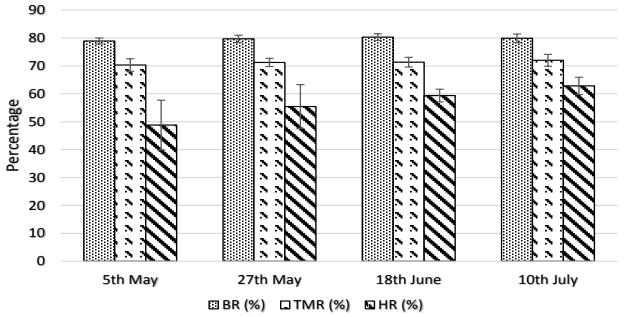

Figure 1: Changes in average brown rice, total milling recovery and head rice recovery percentages of coarse grain rice lines due to changing transplanting dates.

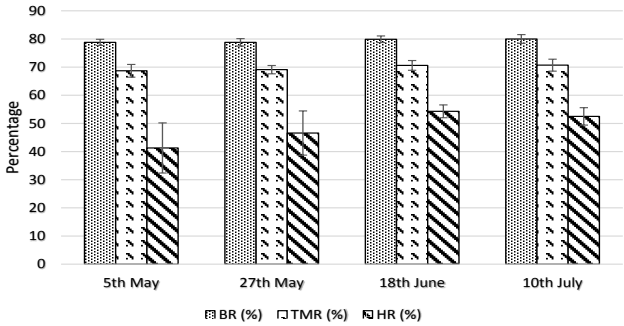

Figure 2: Changes in average brown rice, total milling recovery and head rice recovery percentages of fine grain rice lines due to changing transplanting dates.

genotypes increased from 48.8% to 55.4%, 59.4% and 62.8% from D1, D2, D3 and D4 respectively. In case of fine grain type genotypes, the trend was same for BR and TMR as depicted in Figure 2. For HR%, average HR% increased up to D3 i.e., 54.3% at its maximum, and then decreased after further delaying translating to D4 as depicted in Figure 2. This trend shows that much delay in transplanting after 18th of June, fine grain type rice genotypes may result in more broken grains in final product after milling [13-25].

As depicted in Figure 3, cooked grain length in coarse type rice lines remained nearly stable at all the dates. Cooked grain length increased whereas bursting percentage decreased in fine grain lines with delaying sowing date as depicted in Figure 4. However, in coarse type, bursting percentage decreased drastically and became stable after second date of transplanting. Brown rice percentage and total milling recovery were significantly different among different sowing dates with significant change.

The results in Table 3 shows that on average with respects to date, maximum brown rice (80.3%) was recorded at transplanting date of 18th August 2013 followed by date 10th August 2013 with 79.9% BR. Similarly, maximum TMR (71.3%) was observed transplanting date of 18th August 2013, followed by transplanting date of 27th May 2013. Maximum head rice of 62.8% was observed on transplanting date of 10th July 2013 followed by transplanting date of 18th August 2013 with 59.4% HR. With respect to average data of lines, maximum TMR% of 72.0% was observed for line KSK 469 followed by line PK 7688-1-1-2-2 with 71.9% TMR. Similarly, maximum HR% of 59.8% was observed for line KSK 474 followed by line PK 8785-1-1 with 58.2% HR which is below the HR% of standard check variety of KSK 133 with 62.0% HR.

Date	5th May 2013			27th May 2013			18th June 2013			10th July 2013			Average		
Line/Variety	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)
PK 8785-1-1	78.5	72.5	59	78	69.5	56.7	81	72	58	81	73	59	79.6	71.8	58.2
KSK 474	78	71	52.6	78	70.5	63	81	73	62	77	68	61.5	78.5	70.6	59.8
PK 7688-1-1-2-2	77.4	68.2	46.3	80.1	71.8	51.3	81	73.6	60.5	81	74	65	79.9	71.9	55.8
KSK 469	80	70	33.7	81	73	64.5	80	71	59	81	73	68	80.5	71.8	56.3
KSK 133 (check)	80	73	63	81	73	60	81	72	61.5	81	73	65	80.8	72.8	62.4
KSK 434	80	72	48	80	73	62	78	69	61	81	74	65	79.8	72	59
KSK 462	78	66	42	78	70.7	45.1	80	71	60	79	72	59	78.8	69.9	51.5
KSK 463	80	71	51	80.9	69.6	42.8	79	72	55	80	72	61	80	71.2	52.5
KSK 464	78.3	69	43.5	80	70	53.3	81.7	68.3	57.3	78	69	62	79.5	69.1	54
S.D. (Mean)	± 1.07	± 2.25	± 8.90	± 1.31	± 1.48	± 7.85	± 1.17	± 1.73	± 2.26	± 1.53	± 2.12	± 3.08	-	-	-
Average	78.9	70.3	48.8**	79.7	71.2	55.4**	80.3	71.3	59.4*	79.9	72	62.8*	-	-	-
BR: Brown Rice; TMR: Total Milled Rice; HR = Head Rice															

Table 3: Brown rice, total milling and head rice recovery of different coarse grain rice lines.

Date	1st June 2013			23rd June 2013			14th July 2013			5th August 2013			Average		
Line/Variety	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)	BR (%)	TMR (%)	HR (%)
PK 8660-13-3-1	80	67.5	42	78	68.5	50.5	80	71	51	81	71	52	79.8	69.5	48.9
PK 8662-12-2	78	68	44	79	71.5	55.5	81	74	57	80	70	64.5	79.5	70.9	55.3
Basmati 515	80	72	62	81	74	65	81	68	64	81	74.5	63	80.8	72.1	63.5
PK 8662-20-1-1-1-1	80	71	55.5	78	69	58	81	73	61	81.5	74.5	63.5	80.1	71.9	59.5
PK 8971-24-3-1-19	78	68	35.5	78	68.5	35	81	74	57	80	70	55	79.3	70.1	45.6
PK 8671-24-4-1-20	78.5	68	32.5	80	68	43	80	71	50	79.5	68	38	79.5	68.8	40.9
PK 9118-2-3-1-18	78	68	38	78.5	68	38	79	69.5	47	79	70	48	78.6	68.9	42.8
PK 10052-1	78.5	68	29	78	67	44.5	79	68	50	79	69	35	78.6	68	39.6
EF-1-20-52-04	78.5	68	34	79	68	35	79	69	50	79	71.5	61	78.9	69.1	45
EF-1-30-39-04	78	68	40	78	68.5	41	78	68	56	80	68	45	78.5	68.1	45.5
S.D. (Mean)	± 0.89	± 1.52	± 10.36	± 1.03	± 2.07	± 10.30	± 1.10	± 2.43	± 5.53	± 0.91	± 2.32	± 10.78	-	-	-
Average	78.8	68.7	41.3**	78.8	69.1	46.6**	79.9	70.6	54.3*	80	70.7	52.5**	-	-	-
BR: Brown Rice; TMR: Total Milled Rice; HR: Head Rice															

Table 4: Brown rice, total milling and head rice recovery of different fine grain rice lines.a

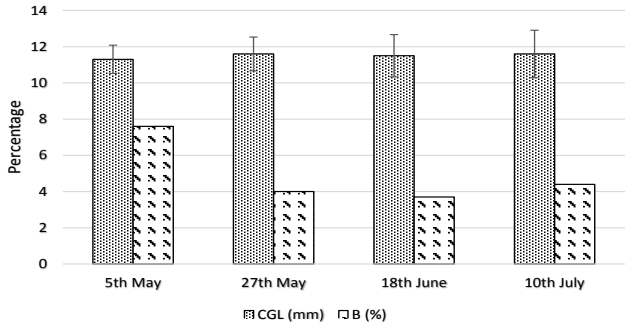

Figure 3: Changes in average cooked grain length and bursting percentages of coarse grain rice lines due to changing transplanting dates.

However, overall line KSK 474 give better result in milling recovery as discussed above.

The Table 4 shows that on average with respect to date of transplanting, maximum brown rice (80.0%) and maximum total milled rice (70.7%) were recorded at transplanting date of 5th August 2013 followed by date 14th July 2013 with 79.9% BR, 70.6% TMR. In similar way, maximum Head Rice (54.3%) was recorded at transplanting date of 14th July 2013 followed by 5th August 2013 with 52.5% HR. Taking average data with respect to lines, maximum TMR (71.9%) was observed for line PK 8662-20-1-1-1-1 followed by line PK 8971-24-3-1-19 with 70.1% TMR. Similarly on average, maximum HR% of 59.5% was observed for line PK 8662-20-1-1-1-1 followed by line PK 8662-12-2 with 55.3% HR. However, this HR% was less than that of our check variety Basmati 515 with 64.5% HR. Individually, maximum HR% of 64.5% was observed for line PK 8662-12-2 on 5th August 2013 followed by line PK 8662-20-1-1-1-1 with 63.5% HR at the same transplanting date. Overall, best milling recovery was observed at transplanting date of 14th July followed by 5th August. None of the under-experiment lines exceeded the check variety of Basmati 515 in terms of milling recovery; however lines PK 8662-12-2 and PK 8662-20-1-1-1-1 gave better results in milling recovery as discussed above [26-30].

The Table 5 shows that on average with respect to date of transplanting, maximum cooked grain length of 11.6 mm with minimum bursting percentage of 4.0% was recorded for transplanting date 27th May 2013 followed by transplanting date 10th July 2013 with 11.6 mm CGL and 4.4% bursting. On average data with respect to lines/variety, maximum CGL of 12.9 mm was observed for line PK 8785-1-1 with 5.0% bursting followed by line PK 7688-1-1-2-2 with 12.8 mm CGL and with 1.5% bursting which is also a minimum. Individually, maximum CGL of 13.6 mm with 5.0% bursting was recorded for line

Date	5th May 2013		27th May 2013		18th June 2013		10th July 2013		Average	
Line/Variety	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)
PK 8785-1-1	12.5	10	13	3	12.8	2	13.4	5	12.9	5
KSK 474	10.2	4	10.5	2	10.4	5	10.2	6	10.3	4.3
PK 7688-1-1-2-2	12	4	12.6	0	13.2	0	13.2	2	12.8	1.5
KSK 469	11	6	11.4	2	10.6	6	12	4	11.3	4.5
KSK 133 (check)	12.2	8	12.5	2	12.8	4	12.7	6	12.6	5
KSK 434	11.2	6	11.5	7	11.8	5	11.7	5	11.6	5.8
KSK 462	11.2	12	11.3	7	11.3	5	11.2	8	11.3	8
KSK 463	11.1	13	10.8	6	10.6	4	10.7	1	10.8	6
KSK 464	10.4	5	10.5	7	10.2	2	9.7	3	10.2	4.3
S.D. (Mean)	± 0.783	± 3.395	± 0.933	± 2.739	± 1.168	± 1.936	± 1.309	± 2.186	-	-
Average	11.3*	7.6**	11.6*	4.0**	11.5*	3.7**	11.6*	4.4**	-	-
CGL: Cooked Grain Length; B: Bursting Upon Cooking										

Table 5: Cooked grain length and bursting parameters of different coarse grain rice lines.

Date	1st June 2013		23rd June 2013		14th July 2013		5th August 2013		Average	
Line	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)	CGL (mm)	B (%)
PK 8660-13-3-1	12.3	3	14.2	2	14.8	2	15.3	1	14.2	2
PK 8662-12-2	11.6	9	13.8	8	13.6	8	14.2	4	13.3	7.3
Basmati 515	12.2	8	14.6	7	15	4	15	5	14.2	6
PK 8662-20-1-1-1-1	12	10	13.4	13	13	12	15.5	0	13.5	8.8
PK 8971-24-3-1-19	12.7	9	14	10	14	4	14.6	4	13.8	6.8
PK 8671-24-4-1-20	13.2	3	15	3	15	0	16.7	0	15	1.5
PK 9118-2-3-1-18	12.5	1	13.5	8	14.3	7	15	4	13.8	5
PK 10052-1	12.6	7	13.1	6	13.8	4	14.5	1	13.5	4.5
EF-1-20-52-04	12.3	60	13	50	12.7	14	13.5	3	12.9	31.8
EF-1-30-39-04	12.5	45	13.8	40	13	6	13.5	4	13.2	23.8
S.D. (Mean)	± 0.428	± 20.04	± 0.636	± 16.446	± 0.853	± 4.332	± 0.960	± 1.897	-	-
Average	12.4	15.5	13.8	14.7	13.9	6.1	14.8	2.6	-	-
CGL: Cooked Grain Length; B: Bursting upon cooking										

Table 6: Cooked grain length and bursting parameters of different fine grain rice lines.

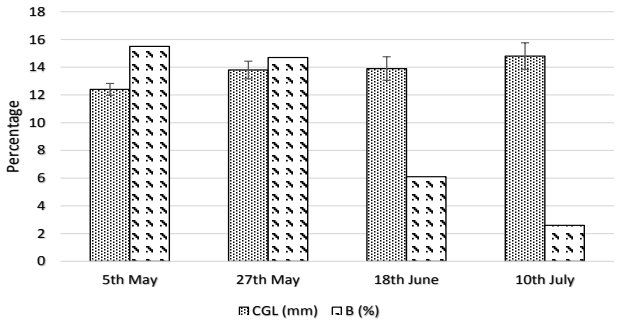

Figure 4: Changes in average cooked grain length and bursting percentages of fine grain rice lines due to changing transplanting dates.

PK 8785-1-1 on 10th July 2013 followed by line PK 7688-1-1-2-2 with 13.2 mm CGL with 0.0% bursting on 27th May 2013. In all, best cooking quality was recorded at 27th May and 10th July and by the lines PK 7688-1-1-2-2 and PK 8785-1-1 performing well as discussed above.

The Table 6 shows that on average with respect to date of transplanting, maximum cooked grain length (CGL) of 14.8 mm with minimum bursting percentage of 2.6% was recorded for transplanting date 5th August 2013 followed by transplanting date 14th July 2013 with 13.9 mm CGL and 6.1% bursting percentage. With respect to average data of lines, maximum CGL of 15.0 mm was observed for line PK 8671-24-4-1-20 with 1.5% bursting which is minimum bursting; followed by line PK 8660-13-3-1 with 14.2 mm CGL and 2.0% whereas check variety Basmati 515 recorded 14.2 mm CGL and 6.0% bursting. As for individual performance, maximum CGL of 16.7 mm was recorded for line PK 8671-24-4-1-20 with 0.0% bursting at transplanting date of 5th August followed by line PK 8660-13-3-1 with 15.3 mm CGL and 1.0% bursting at the same transplanting date. Over all, better cooking quality was recorded at transplanting date of 5th August followed by 14th July. Lines PK 8671-24-4-1-20 and PK 8660-13-3-1 performed well by exceeding the check variety of Basmati 515 in terms of cooking quality parameters as discussed above.

Conclusion

The results showed that the effects sowing date and cultivars on the grain milling and cooking qualities were highly significant. Delayed sowing date, milling quality, total and head rice recovery, cooked grain length and bursting percentages showed different trend with respect to the rice lines. Results suggest that very early transplanting is more damaging to milling and cooking of both, fine as well as coarse grain rice lines as compared to delayed transplanting. Likewise, much delayed transplanting is more destructive for milling and cooking characters in case of fine grain rice lines as compared to coarse grain type rice lines. In case of studied fine grain rice genotypes, head rice recovery was observed maximum when translated at 18th of June. Similarly, cooked grain length was improved significantly in case of fine rice lines with delay in transplanting. Results suggest fine grain rice varieties to be transplanted before onset of July in order to have least broken rice in milled rice. Delayed sowing date, milling quality, total and head rice recovery, cooked grain length and bursting percentages showed

different trend with respect to the rice lines. KSK 133 and Basmati 515 showed maximum head rice recovery among coarse and fine grain rice lines respectively. Likewise, PK 8785-1-1 and PK 8671-24-4-1-20 showed maximum cooked grain length among coarse and fine grain rice lines respectively.

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Nutritional Value of *Macrobrachium rosenbergii* Prawns Fed on Extruded Feeds Enriched with Linseed and Whey Proteins

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Abstract

Three extruded diets were formulated and used to cultivate *Macrobrachium rosenbergii* prawns. For ninety days, the prawns were fed on the similarly balanced isonitrogeneous and isoenergetic diets, based on albumin and linseed (Linseed diet), on whey proteins concentrate (WPC) and soybean oil (Whey proteins diet), and on albumin and soybean oil (Control diet). Every thirty days, the shrimps were weighed and submitted to analyses. The results showed that the average body weights for the shrimps fed on the Linseed and Whey proteins diets were approximately sixty percent higher than for those fed on the Control diet. No significant differences were found for the protein content and the total lipid content in shrimps fed on the diets. However, the Linseed diet led to the lowest cholesterol level and improved the ratio between the sum of n-6 and the sum of n-3 fatty acids in the whole body of shrimps. WPC was used for the first time as a protein source for shrimp feeds, and may be considered as a component to be used in extruded diets for aquaculture. The results suggested that the experimental diets succeeded to provide shrimps with greater weight gain and nutritional value.

Keywords: Cholesterol; Fatty acids; Linseed; Shrimp feed; Whey proteins

Introduction

Population growth, combined with the demand for good-quality seafood, has contributed to the development of shrimp aquaculture. The total annual aquaculture production of all species of freshwater prawns had risen to almost 444000t in 2009 [1]. As an economic activity, the cultivation of the freshwater prawn *Macrobrachium rosenbergii* constitutes 52% of the global total [1]. Its culture has expanded continuously, and reveals that this species has a high potential for aquaculture [2].

Fish meal and fish oil have been the main sources of proteins and lipids in the diets for several cultured specimens [3,4]. In spite of their importance, a coniderable reduction in the use of these ingredients is expected in coming years. Limited availability and variable supply are primary concerns [3]. The availability of fish meal and oil is largely dependent upon weather patterns, and excessive exploitation has caused reduction in supply and increasing prices [3,4]. On the other hand, feed manufacture requires nutritional and cost-effective formulations based on the content and availability of indispensable nutrients, with a reduction in feed cost per unit production [5].

United Nations (UN) Food and Agriculture Organization's (FAO) estimates that 75% of the world's fisheries are fully or overexploited [6]. Replacement of fish meal with alternative sources of proteins from terrestrial animals or plants has been encouraged [6]. Additionally, the use of wild fish in the form of fish meal and fish oil as inputs for aquaculture feeds, relies on marine species that are renewable, but often overexploited for human use [7]. Many studies raise questions about the sustainability of the various alternatives for aquatic feed ingredients. Fish meal and fish oil are limited. Fish oil may in the future be a scarcer commodity than fish meal for use in aqua feeds [7,8].

Supplementation with other ingredients was shown necessary to improve the nutritional quality of such diets [3]. The soluble proteins of milk, also known as whey proteins, are by products of the dairy industry. They have been used in infant and sportsmen formulas because of their high nutritional value, with high levels of essential amino acids, especially branched-chain amino acids. They also have a high level of calcium and bioactive peptides [9,10]. Many positive observations pertaining to the application of whey proteins in wearing pig diets, chickens diets and in calf milk replacers can be found in the literature [11]. Whey proteins are not yet, a competitively priced source of dietary protein for the animal feed industry, with values 7-8 times more expensive than protein from regular soybean meal [11].

Accordingly, the substitution of fish oil in feed compositions has deserved attention. While terrestrial animal fats are rich in saturated fatty acids, vegetal oils are characterized by a high C18-fatty acids content. Recently, experiments were conducted to determine apparent digestibility coefficients of lipid and fatty acids by juvenile halibut. The apparent digestibility of poultry fat (saturated acids) was lower than that of vegetal oils (unsaturated fatty acids). The highest apparent digestibility coefficient was found in groups fed on linseed oil [12]. Linseed oil is considerably good alternative lipid source for salmonids, freshwater fish and prawns. The prices of vegetable oils are more stable and even less expensive than fish oil [13].

Nutritionally, linseed (*Linum usitatissimum*) offers excellent sources of nutrients and energy because it is one of the richest sources of alpha-

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linolenic acid [14,15]. Results from an increasing number of studies clearly indicate that oilseed lipids can be valuable ingredients for fish feed formulations. The replacement of dietary fish oil by vegetable oils had better survival and growth of the freshwater prawn *Mcrobrachium rosenbergii* [16]. Linseed can be included into livestock feeds to modify the fatty acid composition of meat, milk and eggs, providing additional health benefits to consumers [17,18]. Lipids and the associated C-18 polyunsaturated fatty acids (PUFA), linoleic (18:2 n-6) and linolenic (18:3 n-3) acids, as well as n-3 and n-6 highly-unsaturated fatty acids (HUFA) (eicosapentanoic acid, EPA; docosahexanoic acid, DHA; and arachidonic acid, AA), are required in shrimp and other crustaceans feeds [19].

Addition of whey proteins and sources of unsaturated fatty acids, together with other technological resources, may contribute to improve the quality of aquaculture feeds. One of these resources consists of extrusion, which is a process that submits the feed formulation to mixing, shearing and heating under high pressure before the extrudate is forced through a die. Extrusion is the most important process used in the production of fish feeds and pet foods. During the process, feeds undergo reactions that could be beneficial if their nutritional value is improved or detrimental if nutrients are destroyed or become resistant to digestion [20,21]. Studies with blue shrimp (*Litopenaeus stylirostris)* have shown that prior heat treatment was necessary to improve digestibility and possibly inactivate anti-nutritional factors [22]. Denatured proteins are more easily digested compared to the native globular structure. Shear forces play an important role in changing the nutritive value of proteins [21]. This technology also improves the physical characteristics of the pellet, mainly water stability, which is very important for crustacean feeding [22].

Although several studies were reported on partial or complete replacement of fish meal and fish oil in diets for other shrimp species, only a few studies were performed for *Macrobrachium rosenbergii* juveniles with the objective of evaluating their nutritional values. In this work, new extruded feeds enriched with whey proteins and linseed was obtained and the nutritional value of shrimps fed for three months with such diets were evaluated monthly.

Materials and Methods

Defatted egg albumin was purchased from Galgrin Group Ltda. (Caitité, BA, Brazil). Whey protein concentrate (WPC), Alacen™ 450, was supplied by Probiótica Produtos Naturais (São Paulo, SP, Brazil). According to the manufacturer, the concentrate contains 82.3 wt% total proteins. Regular corn starch (CS) with 0.5% gluten, and 12 wt% moisture content was supplied by Corn Products Brazil (São Paulo, SP, Brazil). Food grade butylated hydroxy toluene was supplied by Vetec Química Fina Ltda. (Duque de Caxias, RJ, Brazil). A mixture of minerals and vitamins was purchased from Nestlé Purina (São Paulo, Brazil). Linseed brown was purchased from Erva Viva. Vitamin C was supplied by Hoechst do Brasil Química e Farmacêutica S.A. (Novo Hamburgo, RS, Brazil). A commercial kit CAT. Nº 01400 from Laborlab Produtos para Laboratórios Ltda. (Guarulhos, SP) was used for the quantification of cholesterol in shrimps. All other reagents and solvents were purchased from Vetec Química Fina Ltda. (Rio de Janeiro, Brazil).

Experimental animals and installation

The experiment took place in Itaipuaçu, Maricá, in Rio de Janeiro State (RJ). One thousand juveniles freshwater prawns (*Macrobrachium rosenbergii*), 45-days old, with average initial weight of 130–190 mg, were purchased from Santa Helena Farm, Silva Jardim, RJ. 250 juveniles were separated at the time initial (0 day) to carry out chemical analyses and weighing. Juveniles are very small, requiring a larger number of prawns for chemical analyses. Seven hundred fifty were divided into three groups: 1st, a test group fed on a diet based on egg albumin complemented with linseed (Linseed diet); 2nd, a test group fed on a diet based on whey proteins concentrate and soybean oil (Whey proteins diet); 3rd, a control group fed on a diet based on egg albumin and soybean oil (Control diet). The experiments were performed in polyethylene tanks of 1000 L, covered by a screen. Shelters were provided to serve as refuges. The water in the tanks was aerated with electric pumps (Sarlo Better, model S300–110 V) with a capacity of 280 L/h, and partially changed daily, after being siphoned. In the afternoon, dirt accumulated at the water surface was removed. For 90 days, the prawns were fed in the morning and in the evening with a diet corresponding to 5 wt% of their body weight [23].

Experimental diets and feeding

The diets were formulated according to SEBRAE [23], with the bromathological characteristics recommended for shrimp feed, which consist of protein (25-30 wt%), carbohydrate (30-40 wt%), fat (6-8 wt%), fiber (6-8 wt%), ash (8-10 wt%), moisture (up to 10 wt%), Ca/P ratio (2.5/1). Raw ingredients and formulations compositions are shown in Table 1.

The diet based on linseed was formulated taking into account its cellulose and oil content. In the formulation of the diet based on whey proteins, the lipid content in whey was considered. Linseeds were milled in a conventional blender shortly before processing, and the resulting flour was used.

Preparation of diets

The experimental formulations, with approximately 25 wt% moisture, were prepared by mixing dry ingredients for 30 min in a domestic blender. Diets were prepared by extrusion in a Haake Rheocord 9000 system (Karlsruhe, Germany), equipped with a single-screw extruder, and four heating zones. The barrel length-to-diameter ratio was 25:1. The screw speed was maintained at 20-40 rpm, and

Ingredients (wt. %)	Linseed diet	Whey protein diet	Control diet
Albumin	33.03	-	38.18
Linseed	20.07	-	-
Whey proteins concentrate	-	45.65	-
Corn starch	39.03	36.28	41.15
Soybean oil	-	3.95	6.55
Cellulose	-	6.25	6.25
BHT[a]	0.02	0.02	0.02
Calcium carbonate	2.10	2.10	2.10
Dicalcium phosphate	5.30	5.30	5.30
Minerals and vitamins mixture[b]	0.4	0.4	0.4
Vitamin C[c]	0.05	0.05	0.05
Total	100	100	100

[a] Buthyl hydroxyl toluene (antioxidant); [b] Minerals and vitamins mixture consisting of Mg (0.4 g/kg), Mn (10 mg/kg), Cu (50 mg/kg, Zn (100 mg/kg), I (0.3 mg/kg), Se (0.15 mg/kg), vit. A (3800 IU/kg), vit. D3 (1900 IU/kg), vit. E (140 IU/kg), vit. K (20 mg/kg), folic acid (7 mg/kg), choline (1400 mg/kg), biotin (0.20 mg/kg), niacin (130 mg/kg), calcium pantothenate (40 mg/kg), thiamin (15 mg/kg), riboflavin (20 mg/kg), pyridoxine (20 mg/kg), vit B12 (20 mcg/kg); [c]vit. C (L-ascorbic acid-2-monophosphate (150 mg/kg)

Table 1: Ingredients and composition of experimental diets.

the temperatures of the heating zones were 110, 105, 105, and 90°C from feed zone to die end. The samples were extruded via an attached circular die. After extrusion, the diets were dried to a moisture content around 6 wt% using a forced air oven at temperatures below 55°C.

Chemical analyses

Thirty shrimps (whole body) from each group were individually weighed before the experiments and every 30 days. Although individual weights were recorded, only the average weight values within each tank were used in the data analysis.

The chemical analyses of the shrimp whole body, at different stages of growth (0, 30, 60 and 90 days), were performed in triplicate. On the first day of the experiment, and every 30 days, 70 shrimps were collected from each group to be submitted to analyses. Lipids were extracted from crushed shrimps with chloroform/methyl alcohol/water mixture (1:2:0.8), and quantified according to the Bligh-Dyer method [24]. The protein content was determined by the micro-Kjeldahl method [25].

The enzymatic method (cholesterol oxidase, COD) was used to determine the cholesterol content in shrimps [26,27]. The kit from Laborlab is composed of ready-to-use reagents: a standard reagent (cholesterol solution at 200 mg/dL), enzymatic reagent (lipase ≥ 300 U/mL, COD ≥ 20 U/mL), color reagent 1 (4-aminophenazone at 0.025 mol/L) and color reagent 2 (phenol at 0.055 mol/L). The working reagent was prepared by mixing 0.5 mL of color reagent 1, 0.5 mL of color reagent 2, 19 mL of water, and 0.2 mL of the enzymatic reagent. After lipid extraction and saponification, 2 mL of the working reagent were added to the unsaponifiable fraction. The reaction was carried out in a water bath at 37°C for 10 min. Then, the absorbance of the resulting products was read at 505 nm, in a UV-vis Spectrophotometer Shimadzu model 1601 (Tokyo, Japan), against a control containing only isopropyl alcohol and the working reagent. The cholesterol concentration was determined with a standard curve obtained by plotting absorbance values for standard cholesterol solutions (0.01-0.05 mg/mL).

Fatty acids analyses of diets and whole body of shrimps were carried out on fatty acid methyl esters (FAMEs) diluted in hexane. FAMEs were obtained by base-catalyzed methylation [28]. Separation and analysis of FAMEs were conducted by injecting 1 μL of sample into a Shimadzu gas chromatograph (GC) model QP 2010 (Tokyo, Japan), equipped with a mass spectrometer detector, and a fused-silica capillary column (DB-5ms) from Agilent Technologies (J & W Scientific, CA, USA) with 20 m length, 0.18 μm thickness and 0.25 μm diameter. The GC injector and detector temperatures were set at 250°C; the column oven temperature was set at 110 °C, the column flow was 0.76 mL/min, and the linear velocity was 40 cm/s. Helium was used as the carrier gas at a total flow rate of 42 mL/min. The injector pressure was held constant at 144.0 kPa. The different fatty acids were identified by comparing their mass spectra with the spectra patterns belonging to the library`s equipment. Individual FAME peaks on the gas chromatograms were identified by comparison of retention times.

Fatty acids concentrations were estimated by using the ratio of relative peak area of each fatty acid and the relative peak area of palmitic acid (C16:0), which was the major saturated fatty acid in shrimp samples.

Statistical analysis

The results are presented as mean values ± standard error. The data of different treatments, obtained on every thirty days, were submitted to one-way analysis of variance (ANOVA) with Tukey-Kramer Multiple Comparisons Test for all groups and Student's *t* test unpaired for comparison between two groups. Differences were considered significant at 5%. Statistical analyses were performed by GraphPad InStat 3.01.

Results and Conclusion

The complete substitution of fish meal and fish oil did not affect the survival of *Macrobrachium rosenbergii* juveniles, which was higher than 95%. Also, no negative effect on the palatability of the diets was observed, but an increased feed intake was observed for the diet based on linseed, probably because of its stronger odor.

Although no significant differences in growth rate were observed by Gupta et al. [29] for *Macrobrachium rosenbergii* juveniles fed different experimental isonitrogeneous and isocaloric diets, which varied in terms of percent contribution of major protein sources (fish meal, soybean meal, groundnut oil cake and mustard oil cake), some differences were observed in the present work. Figure 1 shows the variation in weight for the three groups of shrimps, fed for 90 days (Figure 1).

On the 30th day of the experiment, the group fed on the diet based on linseed (0.39 ± 0.06 g) showed a significant improvement in weight ($P<0.05$) in relation to the control group (0.27 ± 0.12 g). This difference disappeared on the 60th day; however, a significant improvement in weight ($P<0.05$) was observed for shrimps fed on the Whey proteins diet (0.58 ± 0.26 g) in relation to the group fed on the Control diet (0.34 ± 0.14 g). On the 90th day, the average weights for the groups fed on the Linseed (0.83 ± 0.28 g) and Whey proteins (0.79 ± 0.19 g) were statistically different ($P<0.05$) in relation to Control diets (0.49 ± 0.12 g). The quality of the lipid profile of linseed might have contributed to the weight gain of shrimps fed on the Linseed diet. Also, the high biological value of proteins of whey (94.6) might have influenced the weight gain of shrimps, compared to the control group, in which the diet was based on egg albumin, with a biological value of 83 [30]. For *Macrobrachium rosenbergii* prawns cultured for 63 days in new earthen ponds to which artificial feed was offered during the last 42 days of culture, the weight gain was higher [31] than those observed in this work. However, for prawns of the same species, fed for 40 days with a diet rich in casein and supplemented with oleic acid and 3.9% lipids, a mean weight similar to those observed for the groups fed on the diets based on linseed and on whey proteins was reported [14]. Anh

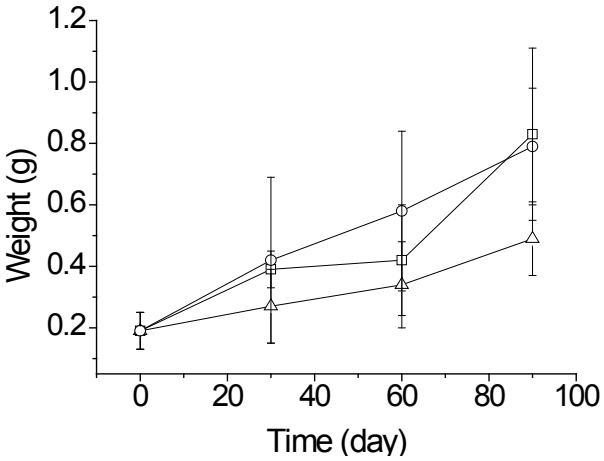

Figure 1: Average weight (g) of *Macrobrachium rosenbergii* prawns along the 90-days experiments; Linseed diet (□), Whey proteins diet (○), Control diet (Δ).

et al. [32] in their studies with diets based on artemia meal, soybean meal, soybean oil, squid oil, gelatin and wheat flour obtained weight values between 0.08- 0.21g, lower to this work, for thirty days. Despite the initial weight being 0.12 g and this work of 0.19 g. Variations in average weight might be attributed to the species, age, availability of food offered to animals (tank size and number of shrimps in the race for food), and type of diet and to the extension of the experiment. In the case of *Macrobrachium rosenbergii* species, males normally occur as three morphotypes, with weights varying in the range 5-250 g [33].

Table 2 shows results from chemical analyses of shrimp whole body in total protein and lipid along the 90-days period.

After 60 days, the group fed on the Whey proteins diet showed a higher protein value (16.37 ± 0.24%) than the other groups fed on the Linseed (11.54 ± 0.49%) and Control (14.98 ± 0.29%) diets (P<0.05). A significant decrease in protein concentration (P<0.05) was observed from the first day up to the 60th day for the group fed on the Linseed diet. However, after 90 days, the protein content for this group was improved and reached a typical value. At the end of the experiments, no statistical difference in protein content was found for the three groups fed on the three different diets, although egg albumin had a lower biological value than whey proteins. For *Litopenaeus vannamei* fed for 8 weeks on a diet based on fish meal, Zhong et al. [34] found a protein content of 16.34 wt%. Protein source was cited as a major factor influencing survival and growth of shrimp [35]. In the present case, the difference in protein source (egg and whey) did not affect survival, and crude protein content in adult *Macrobrachium rosenbergii*. In a study on the growth performance of Pacific white shrimp (*Litopenaeus vannamei*) fed different diets formulated with high levels of soybean meal in combination with 10 wt% either poultry by-product, fish meal, distiller's dried grains with solubles, or pea meal, the diet containing fish meal showed no benefit on growth performance, survival, or feed conversion rate compared with the other formulations [36]. In general, juvenile and sub adult shrimp presented a greater weight gain, percent weight gain, feed efficiency and protein conversion efficiency on a protein-fed basis when fed on a 32% protein diet [37]. Experiment with diets based sardine fishmeal, corn oil, corn flour, wheat bran and soybean meal was found protein content between 13.07–13.95% [38].

As for total lipid, the control group presented the lowest lipid content (1.21 ± 0.11%) (P<0.05) after 30 days. On the 60th day, the groups fed on the Linseed diet (1.63 ± 0.31%) and the Control diet (1.45 ± 0.02%) presented a lower lipid content (P<0.05) than the group fed on the Whey proteins diet (2.21 ± 0.17). However, by incorporating linseed in the diet as the sole source of lipid and fiber, the lipid level was maintained constant until the end of the experiment. After 90 days, the lipid content for whole body of shrimps fed on the Linseed diet was maintained constant along the experimental period. However, no significant differences (P > 0.05) were found for total lipid content in shrimps fed on the different diets. Similarly, for *Litopenaeus vannamei* shrimps fed for 60 days on diets, in which fish meal was replaced by increasing contents of rice protein concentrate [39], no significant difference in tail-muscle composition was found. For several shrimp species, when muscle tissues were investigated [40], the lipid content was lower than those obtained in the present work. Maliwat et al. [38] found lipid content between 0.28–0.76%.

The supposedly high cholesterol content in shrimps has contributed to this crustacean rejection. Consequently, it is important to quantify cholesterol. Figure 2 shows the results found for cholesterol content in whole body, evaluated along the 90-days experimental period.

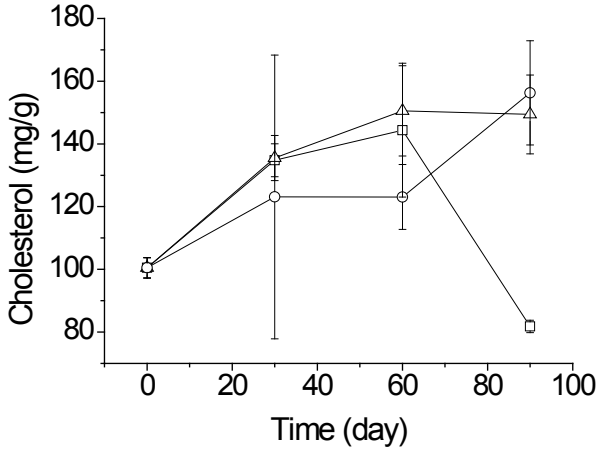

Figure 2: Cholesterol content for whole body of shrimps fed on the different diets along the 90-days experiments; Linseed diet (□), Whey proteins diet (○), Control diet (Δ).

Day	Protein (wt. %)	Lipid (wt. %)
0	15.20 ± 0.14	1.45 ± 0.05
	Linseed diet	
30	14.39 ± 1.03	1.63 ± 0.18
60	11.54 ± 0.49	1.63 ± 0.31
90	14.75 ± 0.58	1.68 ± 0.03
	Whey proteins diet	
30	15.45 ± 0.94	1.83 ± 0.06
60	16.37 ± 0.24*	2.21 ± 0.17*
90	14.90 ± 0.33	1.56 ± 0.01
	Control diet	
30	14.16 ± 1.65	1.21 ± 0.11*
60	14.98 ± 0.29	1.45 ± 0.02
90	14.10 ± 0.42	1.06 ± 0.10

*Superscripts on the same column are significantly different. Significant level (P<0.05) (one-way analysis of variance (ANOVA) with posttest Tukey-Kramer Multiple Comparisons Test)

Table 2: Whole body composition in total protein and lipid from *Macrobrachium rosenbergii* prawns submitted to different treatments along the experimental period.

On the 30th day, a significant increase (P<0.05) in cholesterol content was observed for the shrimps fed on the three diets. Although lower values were determined for shrimps fed on the Whey proteins diet after 30 and 60 days, on the 90th day the highest cholesterol content (156.3 ± 16.6 mg/g) was determined for this group (P<0.05). Contrarily, for shrimps fed on the Linseed diet, after increasing until the 60th day, a significant decrease (P<0.05) in cholesterol content was detected on the 90th day (81.8 ± 1.9 mg/g). This result showed the role of linseed in reducing cholesterol in shrimp tissues. The decrease in cholesterol level found for the group fed on the Linseed diet, compared to the control group and the group fed on the Whey proteins diet may not be attributed to the presence of insoluble fiber in the diet; all the experimental diets had the same amount of fiber. Addition of insoluble fiber does not alter cholesterol level, partly because of hepatic cholesterol synthesis can compensate for poor absorption of cholesterol. Instead, the presence of soluble fibers in diets seems to be responsible for the decrease in cholesterol [41]. Linseed has 10, 22% soluble fiber and 30, 41% insoluble fiber [15]. Soluble fiber may have contributed to the reduction of cholesterol. Viscous fiber could increase intraluminal viscosity and decrease cholesterol content [41]. Also, the hypocholesterolemic effect may be related to the high content

of alpha-linolenic acid (ALA, 18:3n−3), a polyunsaturated fatty acid (PUFA), abundant in linseed [42].

ALA and linoleic acid (LNA, 18:2n-6) and their long-chain derivatives are important components of animal and cell membranes. ALA and LNA are essential polyunsaturated fatty acids (PUFA) that compete for the same enzymes (desaturatases and elongases) to produce their long-chain (20- to 22-carbon atoms) highly unsaturated fatty acids (HUFA) [43]. HUFAs serve as substrates to synthesize eicosanoids (ie, prostaglandins, leukotrienes, prostacyclins, thromboxanes, and lipoxins). The anti-inflammatory properties of eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3) from ALA are known to exert a protective action against atherosclerosis and to have other beneficial effects on human health, such as insulin resistance and blood pressure. On the other hand, a high intake of LNA was appointed to increase inflammatory eicosanoids, and possibly the risk of chronic diseases, and a value of 4.1 or less was recommended for the ratio between Σ n-6 and n-3 [44,45]. An appropriated balance of fatty acids in food was recommended by several authors. Also, because of the limited ability of crustaceans and fishes for de novo synthesis of PUFAs and HUFAs, they need to acquire these EFAs through their diet.

Table 3 summarizes fatty acids ratios determined for prawns at different stages of maturation.

Before the experimental period (on the denoted day 0), palmitic acid (16:0) was the most abundant in whole body of shrimps, followed by elaidic acid (18:1n-9*trans*), stearic acid (18:0), eicosapentaenoic acid (20:5n-3) and linoleic acid (18:2n-6). A small amount of docosahexaenoic acid (DHA, 22:6n-3*cis*) was also detected as FAME by gas chromatography. This result reflected the fatty acid composition of diets offered to early days prawns, conventionally based on fish meal and fish oil.

The content in fatty acids was also quantified periodically along the experimental period. A significant decrease (P<0.05) in the ratio of the sum of n-3 fatty acids (Σ n-3) to palmitic acid was observed for shrimps fed on the soybean oil-rich Control diet. However, a significant increase in the (Σ n-3) to palmitic acid content could be detected along the feeding trial on the Linseed diet. As expected, shrimps fed on the diets supplemented with soybean oil (Whey proteins and Control diets) presented a higher ratio between the sum of n-6 fatty acids (Σ n-6) and palmitic acid than those fed on the Linseed diet. It is worth noting that the amount of soybean oil added to the Whey proteins diet corresponded to about half the amount added to the Control diet. Particularly on the 60th day, the (Σ n-6)/palmitic acid ratio increased significantly (P<0.05) for the shrimps fed on the Control diet (1.3 ± 0.19) in comparison to the result found for the group fed on the Whey proteins diet (0.77 ± 0.05) and the Linseed diet (0.77 ± 0.12).

Days	Σ n-3/16:0	Σ n-6/16:0	Σ n-9/16:0	Σ n-6/Σ n-3	PUFA/SFA
0	1.73 ± 0.28	0.67 ± 0.04	1.67 ± 0.98	0.39 ± 0.09	1.29 ± 0.05
Linseed diet					
30	0.87 ± 0.37	0.64 ± 0.01	1.52 ± 0.07	0.73 ± 0.24	0.87 ± 0.20
60	1.43* ± 0.04	0.77 ± 0.12	1.61 ± 0.06	0.53 ± 0.06	1.23 ± 0.19
90	1.48^{x} ± 0.12	0.73 ± 0.03	2.02* ± 0.02	0.49^{x} ± 0.01	1.15 ± 0.04^{x}
Whey proteins diet					
30	0.42 ± 0.29	1.11 ± 0.38	1.17 ± 0.01	3.84 ± 3.55	0.94 ± 0.12
60	0.17 ± 0.02	0.77 ± 0.05	1,14 ± 0.03	4,76 ± 0,98	0.83 ± 0.39
90	[a]	–[a]	1.20 ± 0.02	–[a]	–[a]
Control diet					
30	0.48 ± 0.16	1.16 ± 0.36	1.18 ± 0.00	2.70 ± 1.66	1.10 ± 0.14
60	0.17 ± 0.12	1.30* ± 0.19	1.03 ± 0.13	9.95 ± 6.12	1.00 ± 0.14
90	0.15 ± 0.00	1.05^{x} ± 0.07	1.00 ± 0.00	7.02 ± 0.57	0.88 ± 0.07

*Indicates statistical differences among means on the same column. Significant level (P<0.05) (one-way analysis of variance (ANOVA) with post-test Tukey-Kramer Multiple Comparisons Test). ^{x}Indicates statistical differences among means. Significant level (P<0.05) (Unpaired t test). [a]Results were not expressed by chromatography. Σ n-3 corresponds to the sum of alpha-linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), eicosatrienoic acid (20:3n-3), docosapentatrienoic acid (22:5n-3) and docosahexaenoic acid (22:6n-3). Σ n-6 corresponds to the sum of linoleic acid (18:2n-6) and arachidonic acid (20:4n-6). Σ n-9 corresponds to oleic acid (18:1n-9)

Table 3: Ratio of Σ n-3, Σ n-6 and Σ n-9 to palmitic acid (16:0), Σ n-6/Σ n-3 and polyunsaurated fatty acids (PUFA) to saturated fatty acids (SFA) determined for the whole body of *Macrobrachium rosenbergii* prawns fed on the different diets.

For whole body of shrimps fed on the Control and the Whey protein diets, the ratio between the sums of n-9 fatty acids (Σ n-9) to palmitic acid was maintained constant along the experimental period. On the other hand, at the end of the trial, this ratio was significantly higher for the group fed on the Linseed diet. For this group, oleic acid (18:1n-9*cis*) was found to be the major fatty acid in whole body of shrimps. As for the ratio between the sum of n-6 fatty acids to the sum of n-3 fatty acids (Σ n-6/Σ n-3), a significantly lower value (P<0.05) was observed at the end of the experiment for the shrimps fed on the Linseed diet in relation to the Control group. On the 90th day, while a (Σ n-6/Σ n-3) of 0.49 ± 0.01 was determined for the group fed on the Linseed diet; this ratio was 7.02 ± 0.57 for the Control group. This result corroborates previous findings related to the nutritional value of linseed oil in reducing Σ n-6//Σ n-3 [42]. Moreover, the high content in linolenic acid present in linseed oil should have contributed to the lowest cholesterol content and the lowest Σ n-6/ n-3 found at the end of the experiment for whole body of shrimps fed on the Linseed diet. Although similar ratios between the contents in PUFAs and saturated fat acids (SFA) were found for the three groups of shrimps in the initial experimental period, this relation was significantly higher (P<0.05) at the end of the experiment for shrimps fed on the Linseed diet (1.15 ± 0.04) than for the group fed on the Control diet (0.88 ± 0.07). According to the British Department of Health [46], to be considered healthy, meat should have a PUFA/SFA value higher than 0.45. In this work, the PUFA/SFA ratios found for shrimps fed on the three diets were characterized as healthy. The PUFA/SFA ratios were higher than 0.45 in shrimp meat.

Despite differences in experimental conditions, commercial freshwater *Macrobrachium rosenbergii* fed on a diet based on hydrolyzed chicken feather and internal organs, fish flour, fish oil and wheat four, the major fatty acids, analyzed by similar methodology, were palmitic acid and eicosapentaenoic acid (EPA). This shrimp was also significantly higher in stearic and elaidic acids than other wild marine species, and presented a PUFA/SFA of 1.19 [40], similarly to the result found in the present work for shrimps fed on the Linseed diet for 90 days. In a 60-day experiment, the growth, survival and nutritional quality in relation to the increased amount of EPA and docosahexaenoic acid (DHA) of freshwater *Macrobrachium rosenbergii* were improved by feeding post larvae with *Moina micrura* enriched with emulsions containing sunflower oil, cod liver oil and commercially available MaxEPA capsules. A maximum EPA of 14.94 ± 0.17% and a maximum DHA of 7.63 ± 0.19% were found [47].

Fish meal and fish oil were successfully substituted by terrestrial proteins and lipids in isonitrogeneous and isocaloric extruded diets for freshwater *Macrobrachium rosenbergii* prawns. Egg albumin was used together with linseed, which was the source of lipids and fiber. A sample of whey proteins concentrate and soybean oil constituted

the base of the second diet. A Control diet was also prepared based on egg albumin and soybean oil. Juveniles fed on the different diets were compared as for weight gain and whole body composition. A high survival was observed, independently of the type of diet. Outdoors conditions, as well as water maintenance and the quality of ingredients could have contributed to this result. The results from this study provided information regarding the application of whey proteins as an alternative source of protein for shrimp diets. Overall, the results suggested that the experimental diets succeeded to provide shrimps with satisfactory growth and healthy nutritional properties.

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The contributors of this book come from diverse backgrounds, making this book a truly international effort. This book will bring forth new frontiers with its revolutionizing research information and detailed analysis of the nascent developments around the world.

We would like to thank all the contributing authors for lending their expertise to make the book truly unique. They have played a crucial role in the development of this book. Without their invaluable contributions this book wouldn't have been possible. They have made vital efforts to compile up to date information on the varied aspects of this subject to make this book a valuable addition to the collection of many professionals and students.

This book was conceptualized with the vision of imparting up-to-date information and advanced data in this field. To ensure the same, a matchless editorial board was set up. Every individual on the board went through rigorous rounds of assessment to prove their worth. After which they invested a large part of their time researching and compiling the most relevant data for our readers.

The editorial board has been involved in producing this book since its inception. They have spent rigorous hours researching and exploring the diverse topics which have resulted in the successful publishing of this book. They have passed on their knowledge of decades through this book. To expedite this challenging task, the publisher supported the team at every step. A small team of assistant editors was also appointed to further simplify the editing procedure and attain best results for the readers.

Apart from the editorial board, the designing team has also invested a significant amount of their time in understanding the subject and creating the most relevant covers. They scrutinized every image to scout for the most suitable representation of the subject and create an appropriate cover for the book.

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The publisher and the editorial board hope that this book will prove to be a valuable piece of knowledge for researchers, students, practitioners and scholars across the globe.

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