SPORTS NUTRITION

STRATEGIES FOR TRAINING, RECOVERY AND PERFORMANCE

NIDHEESH JADEJA

Sports Nutrition: Strategies for Training, Recovery and Performance

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1

Comparison of Site-Specific Bone Mineral Densities between Endurance Runners and Sprinters in Adolescent Women

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Abstract: We aimed to compare site-specific bone mineral densities (BMDs) between adolescent endurance runners and sprinters and examine the relationship of fat-free mass (FFM) and nutrient intake on BMD. In this cross-sectional study, 37 adolescent female endurance runners and sprinters (16.1 ± 0.8 years) were recruited. BMD and FFM were assessed by dual-energy X-ray absorptiometry. Nutrient intake and menstrual state were evaluated by questionnaires. After adjusting for covariates, spine and total bone less head (TBLH) BMDs were significantly higher in sprinters than endurance runners (TBLH, 1.02 ± 0.05 vs. 0.98 ± 0.06 g/cm^2; spine, 0.99 ± 0.06 vs. 0.94 ± 0.06 g/cm^2; $p < 0.05$). There was no significant difference between groups in other sites. The rate of menstrual abnormality was higher in endurance runners compared with sprinters (56.3% vs. 23.8%; $p < 0.05$). FFM was a significant covariate for BMD on all sites except the spine ($p < 0.05$). Dietary intake of vitamin D was identified as a significant covariate only for pelvic BMD ($p < 0.05$). The BMDs of different sites among endurance runners and sprinters were strongly related to FFM. However, the association of FFM with spine BMD cannot be explained by FFM alone. Other factors, including nutrition and/or mechanical loading, may affect the spine BMD.

Keywords: adolescent; sprinters; endurance runners; bone mineral density; fat-free mass; nutrition

1. Introduction

Weight-bearing exercise has positive effects on bone metabolism across the age spectrum [1]. Adolescence is a critical time for bone mineral accrual [2]. Exercises that generate relatively high intensity loading forces enhance bone mineral accretion in adolescents [1]. Thus, adolescent athletes typically have higher bone mass compared with their nonathletic peers [3].

Endurance running has been associated with reduced risks for hypertension, hyperlipidemia, and diabetes [4]. Furthermore, regular running has been reported to reduce proportions of all-cause mortality and disability [5]. However, a subset of adolescent athletes may have impaired bone health [6,7]. Although endurance running is weight-bearing exercise, it has been associated with negative effects on bone in some populations, as indicated by reduced spine bone mineral density (BMD) in endurance runners [8,9]. In contrast, although both sprinters and endurance runners mainly use the lower limbs during exercise, sprinters demonstrate a higher BMD than endurance runners.

The reason for a lower BMD in adolescent female endurance runners may be that this subject group has a greater running distance to cover, higher rate of menstrual irregularities, lower body mass index (BMI), and lower lean tissue mass [6] than sprinters of the same age group. Kusy et al. reported that sprinters in the masters age category have a higher BMD as well as bone mineral content (BMC) at the leg, hip, lumbar spine, and trunk than endurance athletes [10], whereas Bennell et al. reported that differences in the BMD between sprinters and endurance runners (17–26 years) exist only in the lumbar spine [11].

So far, the effect of the ground reaction force has been considered the most significant contributing factor for bone formation [11,12]. However, based on previous studies [10,11], the differences in BMD between sprinters and endurance runners could not be explained solely by the effect of the ground reaction force. Furthermore, although generally higher muscle mass and optimal nutrition is related with increased BMD, the effects of muscle mass and nutrition on BMD among endurance runners and sprinters have not been explored. Recent studies have only focused on BMD in endurance runners [6–9,13]. Clarifying the differences in site-specific BMDs between sprinters and endurance runners may reveal specific factors contributing to BMD gain in sprinters and endurance runners.

The aim of the present study was to compare site-specific BMDs between female adolescent endurance runners and sprinters, and to examine the relationship of fat-free mass (FFM) and nutrient intake with the BMD of different sites.

2. Materials and Methods

2.1. Study Design and Recruitment

In this cross-sectional study, we recruited 37 high school track and field female athletes (16.1 $\pm$ 0.8 years old; competition history of 3.4 $\pm$ 1.9 years), including endurance runners (>800 m, n = 16) and sprinters (100–400 m, n = 21). Study investigators recruited participants from five high schools in the Kansai district of Japan. The study protocol was approved by the Ethics Committee for Human Experiments at Ritsumeikan University (BKC-IRB-2013-031), and was conducted in accordance with the Declaration of Helsinki. All subjects and legal guardians of subjects provided informed consent for participation in this study.

2.2. BMD and Body Composition

We measured the height, body weight, and BMI of each subject. The body mass, fat mass, percent body fat, FFM, and bone mass were evaluated by a dual-energy X-ray absorptiometry (DXA, Lunar Prodigy; GE Healthcare, Tokyo, Japan). During DXA measurements, subjects maintained a supine position. From total body scans, we used enCORE version 15 software (GE Medical Systems Lunar, Madison, WI, USA), which automated measurements of FFM and fat mass (arms, legs, torso, gynoid (gluteal area), and total body), BMD (total bone less head (TBLH), arms, spine, pelvis, and legs), and percent body fat. For screening of at-risk athletes at younger than 20 years for low BMD, TBLH BMD measurement is recommended [14].

2.3. Menstrual State and Stress Fracture History

Menstrual state and stress fracture history were evaluated using questionnaires. For the menstrual state, the age of menarche and characteristics of the menstrual cycle were evaluated. Cycle lengths longer than 45 or shorter than 21 days were considered abnormal [15]. Stress fracture history was defined as having received a diagnosis of stress fracture in a medical institution.

2.4. Food Frequency Questionnaire

A food frequency questionnaire based on the food group (FFQg) was used to estimate usual energy and nutrient intake in athletes. The FFQg estimated nutrient intake from the ingestion frequency and food intake during one week from the most recent 1–2 months [16].

2.5. Physical Activity and Running Distance

Physical activity was estimated from three-day physical activity records. Subjects were instructed to estimate the practice time in minutes.

Running distance was estimated as the mean running distance per one-week from two-week running distance records. Physical activity and running distance were analyzed from the recovered questionnaires (33/37 questionnaires were recovered).

2.6. Statistical Analysis

Statistical analyses were performed with SPSS software version 19.0 (IBM, Tokyo, Japan). All values are expressed as mean ± SD. The independent *t*-test was used to determine differences in physical characteristics, FFM, and BMD between endurance runners and sprinters. An analysis of covariance (ANCOVA) was performed to compare BMD between endurance runners and sprinters, adjusted for age, height, FFM, and fat mass (of total body, arms, torso, gynoid (the gluteal area), and legs), menstrual abnormality, menarche, stress fracture history, and nutrient intake. Those variables that have been reported as important determinant of BMD in previous studies were selected as independent variables [3,6,17,18]. In addition, in a previous study, calcium and vitamin D were chosen as nutrients important for bone health [3]. Of those two nutrients, vitamin D was chosen as covariate, since there was a significant correlation with BMD in the current study. A p value < 0.05 was considered statistically significant.

3. Results

3.1. Subject Characteristics

Table 1 shows the subject characteristics. Endurance runners had a significantly higher running volume than sprinters ($p < 0.01$). Endurance runners also demonstrated a higher incidence of menstrual abnormality ($p < 0.01$) than sprinters. Table 2 shows the physical activities of subjects. Duration of practice was not different between two groups. However, running distance was significantly higher in endurance runners compared with sprinters. Table 3 shows the daily energy and nutrient intake. There was no significant difference between any parameters among the two groups.

Table 1. Subjects characteristics.

	Endurance Runners (n = 16)	Sprinters (n = 21)
Age	16.3 ± 0.8	16.0 ± 0.7
Menstrual abnormality (%)	56.3	23.8 **
Height (cm)	156.7 ±3.7	158.8 ± 4.5
Weight (kg)	47.6 ± 4.6	50.7 ± 5.3
BMI (kg/m^2)	19.4 ± 1.5	20.1 ± 1.9
Fat mass (%)	19.9 ±4.6	19.5 ± 4.4

All values are mean ± SD; **: $p < 0.01$ vs. endurance runners.

Table 2. Physical activity.

	Endurance Runners (n = 14)	Sprinters (n = 21)
Practice time (min/day)	99.6 ± 38.7	109.7 ± 33.1
Running distance (km/week)	58.5 ± 27.1	10.4 ± 5.3 **

All values are mean ± SD; **: $p < 0.01$ vs. endurance runners.

Table 3. Energy and nutrient intake.

	Endurance Runners (n = 16)	Sprinters (n = 21)
Energy (kcal/day)	1927 ± 336	2099 ± 625
Protein (g/day)	70.0 ± 15.1	70.2 ± 20.5
Fat (g/day)	65.1 ± 18.1	78.1 ± 30.3
Carbohydrate (g/day)	258.5 ± 55.6	271.8 ± 78.8
Calcium (mg/day)	582 ± 205	595 ± 270
Magnesium (mg/day)	242 ± 52	232 ± 92
Phosphorus (mg/day)	1052 ± 251	1059 ± 345
Iron (mg/day)	7.5 ± 1.6	7.4 ± 3.1
Vitamin A (μg/day)	578 ± 161	553 ± 210
Vitamin D (μg/day)	6.4 ± 2.9	5.2 ± 2.9
Vitamin K (μg/day)	216 ± 65	182 ± 84
Vitamin B_1 (mg/day)	0.97 ± 0.21	1.00 ± 0.35
Vitamin B_2 (mg/day)	1.14 ± 0.32	1.21 ± 0.41
Vitamin B_6 (mg/day)	1.09 ± 0.22	1.03 ± 0.40
Vitamin B_{12} (μg/day)	6.0 ± 2.3	5.4 ± 2.6
Vitamin C (mg/day)	104 ± 25	88 ± 43

All values are mean ± SD.

3.2. Comparison between Endurance Runners and Sprinters

Table 4 shows subject FFM and BMD values. Endurance runners had a significantly lower FFM in all sites—except for the torso—compared to sprinters. Endurance runners had significantly lower arm, pelvic, spine, and TBLH BMDs than sprinters. However, the leg BMD was not significantly different between endurance runners and sprinters.

In ANCOVA with adjustment for covariates such as age, height, FFM, fat mass, menstrual abnormality, menarche, stress fracture history, and vitamin D intake, the spine and TBLH BMDs remained significantly higher in sprinters than endurance runners ($p < 0.05$) (Figure 1). In contrast, there were no significant between-group differences in other sites.

Table 4. FFM and BMD value among endurance runners and sprinters.

		Endurance Runners (n = 16)	Sprinters (n = 21)
FFM	Arms (kg)	3.2 ± 0.3	3.6 ± 0.4 **
	Legs (kg)	12.5 ± 1.3	13.7 ± 1.1 **
	Torso (kg)	17.0 ±1.7	17.8 ± 1.3
	Gynoid (kg)	5.4 ± 0.5	5.9 ± 0.5 **
	Total body (kg)	36.0 ± 3.2	38.3 ± 2.6 *
BMD	Arms (g/cm^2)	0.767 ± 0.039	0.805 ± 0.038 **
	Legs (g/cm^2)	1.211 ± 0.091	1.262 ± 0.077
	Pelvic (g/cm^2)	1.097 ± 0.086	1.163 ± 0.099 *
	Spine (g/cm^2)	0.942 ± 0.064	0.985 ± 0.062 *
	TBLH (g/cm^2)	0.981 ± 0.061	1.023 ± 0.050 *

All values are mean ± SD; FFM: fat-free mass, BMD: bone mineral density, Gynoid: the gluteal area, TBLH: Total Bone Less Head; **: $p < 0.01$, *: $p < 0.05$ vs. endurance runners.

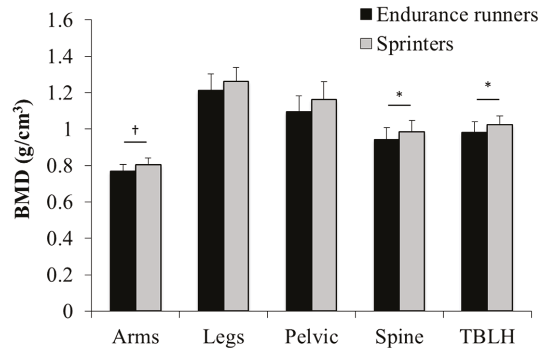

Figure 1. Comparison of adjusted BMD between endurance runners and sprinters. Endurance runners vs. sprinters; Spine: 0.94 ± 0.06 vs. 0.98 ± 0.06 g/cm^2, TBLH: 0.98 ± 0.06 vs. 1.02 ± 0.05; *: $p < 0.05$, †: $p = 0.06$.

3.3. Effect of Covariates on the BMD of Different Sites

In ANCOVA, FFM was a significant covariate for arms ($p < 0.01$), legs ($p < 0.05$), and pelvic ($p < 0.05$) BMD, and tended to be a covariate for TBLH BMD ($p = 0.05$) (Table 5). Additionally, vitamin D intake was identified as a significant covariate for arms ($p < 0.05$), pelvic ($p < 0.01$), and spine ($p < 0.05$) BMD, and tended to be a covariate for TBLH BMD ($p = 0.05$). Moreover, menarche was a significant covariate for arms BMD ($p < 0.05$).

Table 5. Multivariable linear regression model on BMD of all subjects.

	Arms	Legs	Pelvic	Spine	TBLH
Age	0.48	1.74	2.61	0.06	0.48
Height	0.34	0.06	0.01	0.00	0.06
FFM (each site)	11.37 **	4.83 *	7.49 *	0.05	4.13 †
Fat-mass (each site)	0.86	0.10	3.45	0.21	0.25
Menstrual abnormality	0.40	2.05	0.14	1.31	0.86
Menarche	6.13 *	1.78	0.17	1.04	1.61
Stress fracture history	2.00	0.97	0.18	0.88	1.29
Vitamin D intake	4.82 *	1.49	8.08 **	4.31 *	4.04 †

All values are *F* values; **: $p < 0.01$, *: $p < 0.05$, †: $p = 0.05$.

4. Discussion

The purpose of this cross-sectional study was to compare BMDs of various sites and examine the association with different factors on the BMD among female high school track and field athletes. The main finding of our results was that endurance runners had significantly lower BMD in spine and TBLH as compared with sprinters, even after adjusting for covariates. In addition, vitamin D intake seems to have a site-specific association with arms, pelvic, and spine BMD. Furthermore, FFM was a significant covariate for most BMDs, with the exception of the spine.

4.1. The Difference between the BMD of Sprinters and Endurance Runners

When comparing the BMD of sprinters and endurance runners using a *t*-test, the BMDs of the arms, pelvis, spine, and TBLH in sprinters were significantly higher than those in endurance runners. However, after adjusting for covariates, between-group differences remained significant only for spine and TBLH BMDs.

In a previous study, ground reaction force with foot-strike during running was reported to be 1–2 times the body weight for low intensity forms of running (e.g., endurance) while it becomes 2–4 times the body weight for high intensity forms of running (e.g., sprint) [19]. According to the mechanostat theory, an increase in the bone mass is caused by larger bone deformation (e.g., high ground reaction force) which exceeds the normal strain for modeling [20]. However, the ground reaction force decreases as it is transmitted upward to the pelvis and spine from legs [13]. Since endurance runners experience smaller ground reaction force than sprinters, endurance runners may have less loading and deformation to spine bone when compared with sprinters with higher ground reaction forces. Additionally, in a previous study, weekly running volume was inversely correlated with lumbar spine BMD [21,22]. Greater running distance results in large energy expenditures, and one possible explanation for its effect on bone is via a potential catabolism when energy intake was insufficient, leading to low energy availability. Low energy availability has been shown to increase bone resorption and decrease bone formation, potentially mediated by reduced levels of insulin-like growth factor 1 or estradiol, resulting in low BMD [23,24]. Trabecular bone such as spine has been shown to be easily influenced by low energy availability [13]. Average running volumes of previously reported studies were 68.4 ± 12.1 km/week [21] and 32 ± 8 km/week [22] for endurance runners. Our current study participants exercised 58.5 ± 27.1 km/week among endurance runners and only 10.4 ± 5.3 km/week among sprinters, while their energy intake was identical between groups. Therefore, the low BMD of endurance runners may have been caused by both smaller mechanical loading as well as less energy availability as compared with sprinters.

Previous studies comparing the BMD of endurance runners and sprinters have often shown a difference in the leg BMD between the two groups [11,25]. However, this difference was not observed in the present study. The reason for this difference may be related to the subjects' age and competition history. In a previous study, the subjects were over 17 years of age, and they had a competition history of over a decade [25]. In addition, in a previous study comparing the BMD of 13- to 18-year-old runners and non-runners, when separated by age, runners had significantly lower total body BMD compared with non-runners in the 17- to 18-year-old age group, but no difference was observed among groups of 13- to 16-years old [13]. The subjects in the present study had a mean age of 16.1 ± 0.8 years and mean competition history of 3.4 ± 1.9 years. Therefore, the lack of observed difference in leg BMD among long distance runners and sprinters may be caused by their age (bones being still in the growth stage) and relatively short competition history.

4.2. Relationship between Muscle Mass and BMD

After adjusting for age, height, FFM, fat-mass, menstrual abnormality, menarche, stress fracture history, and vitamin D intake, there were no significant group differences in the BMDs of the arms, legs, and pelvis. Among these confounding factors, FFM had the greater F value at each site. Thus, the FFM could have a strong influence on the BMDs among all sites. However, FFM was not found to be a significant covariate for spine BMD, while vitamin D intake was a significant covariate. Therefore, these results suggest that the spine might be more affected by nutrient factors such as vitamin D.

The close relationship between muscle mass and bone mass has been known for a long time [26]. In a previous study, sprinters were shown to have a higher FFM than endurance runners. Kusy and Zielinski [10] demonstrated that greater skeletal size allows exertion of larger muscle forces, supporting engagement in sprint disciplines, or forces exerted during sprinting induce skeletal adaptation and augment BMD. In addition, in a longitudinal study of 68 children (8 to 14 years), the maximal increase in lean body mass (LBM) occurred a several months before the maximal increase in BMC, indicating a close relationship between muscle and bone development [27]. These findings suggest that among adolescent female track and field athletes in their growth period, sprinters may have higher FFM and exercise intensity than endurance runners. Thus, in accordance with mechanostat theory, sprinters demonstrate higher BMDs than endurance runners.

4.3. Effect of Site-Specificity in Vitamin D

Vitamin D intake seems to have a site-specific relationship with arms ($p < 0.01$), pelvis ($p < 0.05$), and spine ($p < 0.05$) BMDs. A previous study using a vitamin D analogue indicated that the effect with vitamin D differs between cortical and trabecular bone. Takahashi et al. [28] concluded that vitamin D compounds might suppress receptor activator of nuclear factor-kappa B ligand (RANKL) activity in superficial osteoblastic cells of the trabecular bone. RANKL is an essential cytokine for activating osteoclast (increase in bone resorption). Therefore, habitual high vitamin D intake has a potential positive effect on pelvic and spine BMDs of trabecular bone. On the other hand, vitamin D intake was identified as a significant covariate for arms BMD. The bone of the arms consists mostly of cortical bone, since it is long bone. Thus, the aforementioned explanation for vitamin D and trabecular bone may seem inconsistent. However, FFM and menarche were demonstrated as significant covariates for arms. Since running puts minimal mechanical stress on arms, other variables such as FFM and nutrients may have had a larger influence. In the present study, the strongest covariate for arms BMD was FFM ($F = 11.37$, $p < 0.01$). However, since the results of the present study cannot explain the causal relationship, further study is warranted.

4.4. Study Limitations

This study included a relatively small sample size. Furthermore, the causal relationship cannot be determined by the current cross-sectional study without an inactive control group. Several parameters were not evaluated, such as bone metabolism markers, sex hormones (e.g., estrogen and progesterone), and reproductive maturation (such as tanner breast stage). Moreover, a previous study reported that subclinical ovulatory disturbance provides negative effect on bone [29]; however, the present study did not assess that. Low-dose oral contraceptives may impair the attainment of peak bone mass [30]. It should be noted, however, that the subjects of the present study were not taking oral contraceptives. In addition, FFQs for dietary assessment have been validated on collegiate woman, and not with the same age group of subjects in the current study. Accordingly, the dietitians used food samples to demonstrate the correct portion of specific foods. Future prospective studies are needed in other populations to determine variations, and intervention studies are warranted to determine the effects of FFM and vitamin D on site-specific BMDs.

5. Conclusions

We conclude that differences in the BMDs of different sites among endurance runners and sprinters were strongly affected by FFM. Furthermore, vitamin D intake also seems to have site-specific associations with BMDs. However, the relationship of FFM on spine BMD cannot be explained by FFM alone. Other variables, including nutrition (e.g., vitamin D) and/or mechanical loading may have been associated with spine BMD.

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Author Contributions: Aoi Ikedo, Kumiko Ebi and Satoshi Fujita conceived and designed the experiments; Aoi Ikedo, Aya Ishibashi, Saori Matsumiya and Aya Kaizaki performed the experiments; Aoi Ikedo analyzed the data; Aoi Ikedo, Aya Ishibashi, Saori Matsumiya, Aya Kaizaki contributed reagents/materials/analysis tools; Aoi Ikedo and Satoshi Fujita wrote the paper.

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2

Probiotic Supplements Beneficially Affect Tryptophan–Kynurenine Metabolism and Reduce the Incidence of Upper Respiratory Tract Infections in Trained Athletes: A Randomized, Double-Blinded, Placebo-Controlled Trial

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Abstract: Background: Prolonged intense exercise has been associated with transient suppression of immune function and an increased risk of infections. In this context, the catabolism of amino acid tryptophan via kynurenine may play an important role. The present study examined the effect of a probiotic supplement on the incidence of upper respiratory tract infections (URTI) and the metabolism of aromatic amino acids after exhaustive aerobic exercise in trained athletes during three months of winter training. Methods: Thirty-three highly trained individuals were randomly assigned to probiotic (PRO, n = 17) or placebo (PLA, n = 16) groups using double blind procedures, receiving either 1×10^{10} colony forming units (CFU) of a multi-species probiotic (*Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63, and *Lactococcus lactis* W58) or placebo once per day for 12 weeks. The serum concentrations of tryptophan, phenylalanine and their primary catabolites kynurenine and tyrosine, as well as the concentration of the immune activation marker neopterin were determined at baseline and after 12 weeks, both at rest and immediately after exercise. Participants completed a daily diary to identify any infectious symptoms. Results: After 12 weeks of treatment, post-exercise tryptophan levels were lowered by 11% (a significant change) in the PLA group compared to the concentrations measured before the intervention (p = 0.02), but remained unchanged in the PRO group. The ratio of subjects taking the placebo who experienced one or more URTI symptoms was increased 2.2-fold compared to those on probiotics (PLA 0.79, PRO 0.35; p = 0.02). Conclusion: Data indicate reduced exercise-induced tryptophan degradation rates in the PRO group. Daily supplementation with probiotics limited exercise-induced drops in tryptophan levels and reduced the incidence of URTI, however, did not benefit athletic performance.

Keywords: intense exercise; kynurenine; tryptophan; probiotics; upper respiratory tract infections

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1. Introduction

Numerous studies have shown that prolonged intense physical exercise is associated with a transient depression of immune function in athletes. While moderate exercise beneficially influences

the immune system [1], a heavy schedule of training and competition can lead to immune impairment associated with an increased risk of upper respiratory tract infections (URTIs) due to altered immune function [2,3]. It has been suggested that exhaustive exercise creates a potential 'open window' of decreased host protection, during which viruses and bacteria can gain a foothold, increasing the risk of developing an infection [4]. During major competitions of 2–3 weeks duration, typically about 7% of athletes experience at least one episode of illness and about half of these are respiratory [5]. Exercise immunological studies reported that infection episodes were preceded by declines in immunoglobulin A (IgA) in saliva [6–8]. Furthermore, results suggest a possible mechanism for the increased incidence of infection during intensified training via modulation of type 1/type 2 T lymphocyte distributions [9].

Physical exercise and sports influence immunoregulatory circuits which, as a primary response, involve the production of forward regulatory cytokines is followed by counter-regulation leading to an immunosuppressed state [3,10,11]. Downstream biochemical events include changes in tryptophan (Trp) metabolism when T helper cell type 1 (Th1-type) cytokine interferon-γ (IFN-γ) is released and induces tryptophan-degrading enzyme indoleamine 2,3-dioxygenase (IDO-1). In turn, blood concentrations of Trp become reduced, leading to various potential consequences [12]. The essential amino acid Trp is not only a precursor of the serotonin biosynthesis pathway but is also the key element for the formation of the energy carrier and coenzyme nicotinamide-adenine-dinucleotide NAD and its reduced form NADH via the so-called kynurenine (Kyn) pathway [13,14]. Recently, exhaustive aerobic exercise in athletes was reported to significantly impact on Trp–Kyn metabolism [15]. Results indicate an involvement of IDO-1 activation in enhanced Trp catabolism and Kyn production following demanding exercise [15]. The close association of Trp metabolites with neuropsychopharmacologically relevant metabolites may have special consequences for athletes since it influences immunosurveillance and the development of infections as well training adherence because of disturbed neurotransmitter biochemistry [16].

Trp is also an important target for the gut and brain interaction [17]. In addition to its resorption from dietary components, the composition of gut bacteria—the microbiome—is of enormous importance in the regulation of Trp. Available data suggest a role for the gut microbiota in actually modulating Trp and hence having control over serotonin levels in the host [18]. Recently, an inverse correlation of serum levels of Trp, tyrosine, and phenylalanine with concentration of fecal calprotectin, a marker for gut leakiness, has been reported in patients suffering from Alzheimer's disease, thus indicating a close relationship between the intestinal barrier function and aromatic amino acid concentration in the blood [19]. Furthermore, there is growing body of evidence indicating that the microbiota is sensitive to physiological changes associated with exercise [20,21]. For example, acute aerobic exercise reduces the expression of toll-like receptors (TLRs) in the monocyte cell-surface, contributing to post-exercise immunodepression, while over the long-term, a decrease in TLR expression may represent a beneficial effect because it decreases the inflammatory capacity of leukocytes, thus altering whole body chronic inflammation [22]. TLRs can activate dendritic cells, which are associated with the attenuation of immune activation and inflammation protection [20]. Notably, IDO-1 has been identified in mucosal Cluster of Differentiation 103 -expressing dendritic cells and has already been claimed to be a possible therapeutic target for gut disorders [23].

Dietary supplements containing probiotics can modify the population of the gut microflora and may provide a practical means of enhancing gut and systemic immune function, which was shown to be beneficial by reducing the infection frequency in sensible groups, e.g., elderly in group homes or children [24,25]. However, studies in these subject groups might not be reflective of athletes who have different gut microbiota [26]. Exercise and associated dietary extremes were shown to increase gut microbial diversity in comparison to sedentary people [27]. Some studies have established that probiotic intake can improve low-grade inflammation [28,29] and enhance resistance to URTI in athletes [30–32]. In a previous study, Lamprecht and colleagues found that adequate probiotic supplementation composed of six strains consisting of *Bifidobacterium bifidum* W23,

Bifidobacterium lactis W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63, and *Lactococcus lactis* W58 could improve redox hemostasis and low-grade inflammation in men under sustained exercise stress [29]. The mechanisms behind these observations have not been widely investigated but may include direct interaction with gut microbiota, interaction with mucosal immune system and modulation of lung macrophage and T cell functions [33]. For example, one study observed that the IFN-γ response (a potent stimulus for IDO-1) was moderately higher with probiotic treatment than with placebo, associated with a significant reduction in the number of days of respiratory illness symptoms in highly trained distance runners [30]. Since Trp availability is primarily regulated via the Kyn pathway, the catabolism of amino acid Trp via Kyn may play an important role on the risk of developing an infection.

The aim of the present study was to examine the effect of a probiotic supplement on the incidence of URTI and Trp metabolism after exhaustive aerobic exercise in trained athletes during three months of winter training We hypothesized that daily supplementation with probiotics is beneficial in reducing the incidence of URTI in athletes during training periods in winter and is associated with modulation of the Trp—Kyn metabolic pathways.

2. Materials and Methods

2.1. Subjects

Thirty-three healthy and trained volunteer athletes (mean age 26.7 years; average body mass index 22 kg/m^2; average peak oxygen uptake 51.4 mL/kg/min) participated in this study that was conducted at the Department of Sport Science at the Leopold Franzens University of Innsbruck, Austria. Individuals were invited to participate if they were 20–35 years of age, non-smokers, had no previous history of muscle disorders and were free of heart, kidney, lung, neurologic, and psychiatric diseases. Athletes with a cardiorespiratory response and fitness of $\geq$150% of reference values during maximal exercise [34] were included. A questionnaire about medical history and previous training was filled out by each participant. In total, 33 individuals were enrolled with 29 participants (13 men 16 women) completing the study. Baseline characteristics of the subjects are presented in Table 1.

Table 1. Baseline characteristics, nutrition and performance data of the participants.

Variable	Unit	Probiotics (*n* = 14) Mean ± SD	Placebo (*n* = 15) Mean ± SD
Gender	male/female	8/6	5/10
Age	year	25.7 ± 3.5	26.6 ± 3.5
BMI	kg/m^2	22.2 ± 1.5	21.2 ± 2.7
Weight	kg	67.4 ± 9.6	62.9 ± 11.1
Body cell mass	kg	31.2 ± 6.6	28.7 ± 7.4
Total body fat	%	20.1 ± 5.7	19.5 ± 4.4
VO_{2max}	mL/kg/min	55.1 ± 6.4	47.5 ± 7.1 **
P_{max}	watt	325 ± 54.2	274 ± 51.6 *
P_{rel}	watt/kg	4.8 ± 0.3	4.3 ± 0.4 **
P_{TT}	watt	222 ± 41.9	181 ± 38.3 *
Energy intake	kcal/day	2821 ± 1374	2840 ± 1161
REE	kcal/day	1602 ± 206	1519 ± 2031
Protein	%	14.9 ± 3.3	15.0 ± 3.5
Carbohydrates	%	49.5 ± 12.4	49.3 ± 12.7
Fat	%	32.5 ± 10.8	33.0 ± 12.1
Fibers	g	33.0 ± 10.1	32.0 ± 14.2
Alcohol	g	11.1 ± 10.7	9.4 ± 9.5
Water	L	3.38 ± 0.58	3.37 ± 0.84

Values are means ± SD; Significant difference between the groups: * $p < 0.05$; ** $p < 0.01$; BMI: body mass index; VO_{2max} = peak oxygen uptake; P_{max} = peak power output; P_{rel} = peak power output related to body weight; P_{TT} = Time-trial power output; REE = resting energy expenditure.

Subjects who met the inclusion criteria of the study were randomly assigned to the treatment or placebo group. The randomization code was held by a third party and handed over for statistical analyses after collection of all data. All of the participants were informed of the risks and potential discomforts associated with the investigation and signed a written consent to participate. The study was approved by the Board for Ethical Questions in Science Ethics at the Leopold Franzens University of Innsbruck according to the principles expressed in the Declaration of Helsinki.

2.2. Study Intervention

Subjects randomized to probiotics (PRO, n = 17) received boxes with sachets containing multi-species probiotics composed of six strains consisting of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63, and *Lactococcus lactis* W58 (Ecologic® Performance, Winclove B.V., Amsterdam, The Netherlands). The total cell count was adjusted to 2.5×10^9 colony forming units (CFU) per gram. The candidate strains were selected upon their survival in the gastrointestinal tract, activity, intestinal barrier function, and anti-inflammatory properties and were used in a previous study on immune health in athletes [29]. The matrix consisting of cornstarch, maltodextrin, vegetable protein, $MgSO_4$, $MnSO_4$ and KCl. Subjects were instructed to take 1 sachet of 4 g per day, which is equivalent to 1×10^{10} CFU/day, with 100–125 mL of plain water, one hour prior to breakfast and throughout the 12 weeks. Those subjects assigned to the placebo group (PLA, n = 16) received identical boxes and sachets with the same instructions for use.

2.3. Study Protocol

During the three-month intervention period (January 2015 to March 2015) subjects were asked to maintain their normal diet and to continue with their normal training programs. In addition, participants agreed to avoid taking medicine including anti-inflammatory drugs (e.g., aspirin, ibuprofen, voltaren) and antibiotics, additional probiotics and dietary supplements such as fish oil, vitamins (vitamin C, vitamin E) and minerals (selenium). Consumption of alcohol (>10 and 20 g for women and men, respectively, per day), or any fermented dairy products (e.g., yoghurt) was not permitted during this period. During the first visit to the laboratory, measures of participants' weight and height were obtained using standardized methods and used to calculate body mass index (BMI, kg/m^2). Prior to and at the end of the study, all subjects were tested for body fat (in percent of body weight), body cell mass (kg), and resting energy expenditure (kcal/day) using the bioelectrical impedance analysis (BIA) method (BIA-2000-M, Data Input, Pöcking, Germany). Prior to the first blood draw and after 12 weeks of supplementation, participants were asked to complete a three-day food record to evaluate energy and nutrient intake. Diet records were analyzed for total calories, protein, carbohydrate, fat, alcohol, and water intake using "nut.s science" nutritional software (dato Denkwerkzeuge, Vienna, Austria). Weekly training (modality, frequency, intensity, volume) and illness (URTI symptoms and gastrointestinal GI complaints symptoms) logs were kept.

The illness symptoms listed on the self-constructed questionnaire, modified according to Gleeson et al. (2011) [31] were sore throat, runny nose, cough, fever, and weakness. Subjects were asked to rate the severity of their symptoms (very light, light, moderate, severe, very severe). The GI discomfort symptoms listed on the questionnaire were abdominal pain, diarrhea, loss of appetite, vomiting, and others. The incidence score relates to the number of participants who reported symptoms in each arm of the study. One or more symptoms on at least two consecutive days were defined as an episode of illness. Symptoms with an interval of only one day were counted as the same episode.

2.4. Exercise Tests

In the morning of the exercise test a standardized breakfast was provided 2 h prior to strenuous exercise tests (379 kcal; 88 energy percent carbohydrates, 11 energy percent proteins, and 1 energy percent fat). The composition of this standardized breakfast is shown in Table 2.

Table 2. Composition of the standardized breakfast 2 h prior to strenuous exercise tests.

Food	Energy (kcal)	Protein (g)	Carbohydrates (g)	Fat (g)
2 wheat rolls 100 g	260	8.70	52.7	0.90
Marmalade/jam 50 g	114	0.30	28.0	0.00
250 mL tea	5	0.75	0.25	0.25
250 mL water	-	-	-	-
Total	379	9.75	80.95	1.15
Meal energy (%)		11	88	1

For eligibility testing all subjects performed an incremental cycle ergometer exercise test until exhaustion. Cycle ergometry was performed on an electronically braked ergometer (Ergometrics 900, Ergoline, Germany) and started at a workload of 50/75 W (women/men) for 5 min (warm up) with a following increase in workload of 25 W per minute until exhaustion. Exhaustion was defined as the state when the pedaling rate dropped below 60 rpm. Heart rate and ventilatory parameters were monitored continuously (Oxycon mobile, Jaeger, Germany). Peak power output (P_{max}) was defined as the last completed workload rate plus the fraction of time spent in the final uncompleted work rate multiplied by 25 W [35]. Peak oxygen uptake (VO_{2max}) was defined as the highest 30-s average during the test.

After a 20 min resting period, athletes performed a 20-min maximal time-trial on a cycle ergometer (RBM Cyclus 2, Leipzig, Germany) as described by Faulhaber and colleagues [35]. Briefly, the cycle ergometer was shifted to a fixed pedal force in which power output was dependent on the pedaling rate. Pedal force for each participant was set so that pedaling at 100 rpm would produce about 70% (rounded to 5 W) of peak power output, which was determined by the incremental cycle ergometry. During the test, cyclists were strongly encouraged to choose a maximal pedaling rate that could be maintained for the respective test duration. The main outcome measurement was mean power output during the 20-min test, which was automatically calculated by the software of the ergometer. The participants were allowed to drink water ad libitum. Three months later this procedure was repeated on the same cycle ergometer and with the same investigator.

2.5. Blood Measurements

We conducted blood collections from the participants in the supine position from a medial cubital vein at baseline and after 12 weeks at rest and within 5 min after exercise (four blood draws per study participant). After centrifugation for 10 min cells were removed and plasma samples were frozen at −20 °C until analysis. Serum concentrations of Trp and Kyn as well as concentrations of phenylalanine (Phe) and tyrosine (Tyr) were determined by high-performance liquid chromatography (HPLC), as previously described [36,37]. The ratios of Kyn/Trp and Phe/Tyr were calculated as indexes of Trp degradation and phenylalanine 4-hydroxylase (PAH) activity, respectively. Pro-inflammatory cascades were found to be associated with disturbed PAH activity [37]. Serum neopterin concentrations were measured by ELISA (BRAHMS Diagnostics, Hennigsdorf, Germany) following the manufacturer's instructions [38].

2.6. Statistical Analysis

Per protocol analyses were performed using SPSS (IBM SPSS Statistics Version 22, IBM Corp., Armonk, NY, USA). Normality in the distribution of data was tested using the Kolmogorov-Smirnov's test and Boxplots. In the case of Gaussian distribution, baseline characteristics, performance data, nutrient and biological markers were compared by unpaired Student's *t*-test or Mann-Whitney-*U*-Test. Changes in variables during the study were analyzed by univariate analysis of variance (ANOVA) for parametric variables. The Wilcoxon-signed rank and Friedman test were applied to non-parametric data. Spearman's rank correlation was used to assess the association between two variables. Partial eta-squared values were calculated to estimate the effect of any statistically significant

differences found. Using the guidelines of Cohen [39], 0.01 = small effect, 0.06 = moderate effect, and 0.14 = large effect. A p-value of less than 0.05 (two-tailed) was considered to indicate statistical significance. Data are presented as mean values ± standard deviation (SD) or by mean values ± standard error of the mean (SEM).

Sample size calculation was based on changes in exercise-induced Trp levels [40] from baseline to the end of the 12-week intervention between the PRO group and the control. We estimated between 10 and 12 subjects per group—depending on SD and effect size—to reach a probability of error (alpha/2) of 5% and 80% power. Allowing for a drop-out rate of 30%, 16 subjects per group were recruited.

3. Results

3.1. Study Population

Twenty-nine of the 33 randomized subjects completed the full program and entered statistical analyses. Three withdrew because of injury or persistent illness with antibiotic medications, one because of a longer training interruption. Returned sachet count after the treatment period revealed a compliance rate >95% in both groups (97.6% in the probiotics group, 98.8% in the control group). The lowest level of compliance for a subject was 86.9%. A CONSORT (Consolidated Standards of Reporting Trials) diagram outlining participant recruitment is depicted Figure 1.

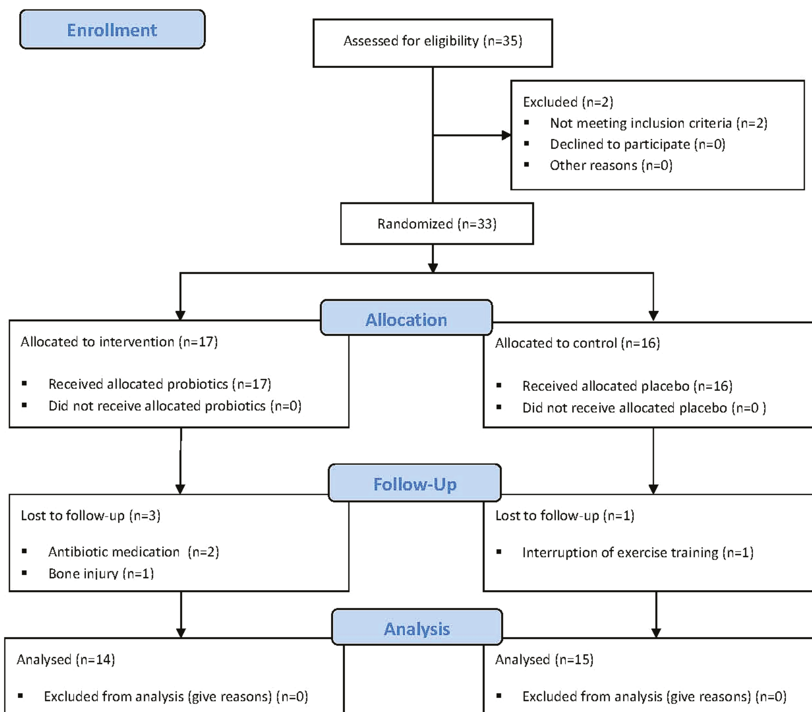

Figure 1. Flow of participations through each stage of the trial.

At baseline, a significant gender-dependent difference (females were overrepresented in the control group), VO_{2max} and Trp was observed between groups ($p < 0.05$). Females had a lower BMI, VO_{2max}, and mean power output during the 20-min test (P_{TT}) compared to male athletes, as Kyn levels were lower in females ($p = 0.019$). None of the other parameters were influenced by gender.

3.2. Training Loads

Analysis of training loads indicated that the weekly training of the aerobic system, mainly continuous endurance training at moderate intensity (60% to 80% VO_{2max}), varied significantly between the groups over the 12-week treatment period (Figure 2). The means were significantly higher in the probiotics group as compared to the placebo group: 8.0 ± 2.3 and 6.6 ± 4.3 h per week endurance training, respectively ($U = 2.597$, $p < 0.001$).

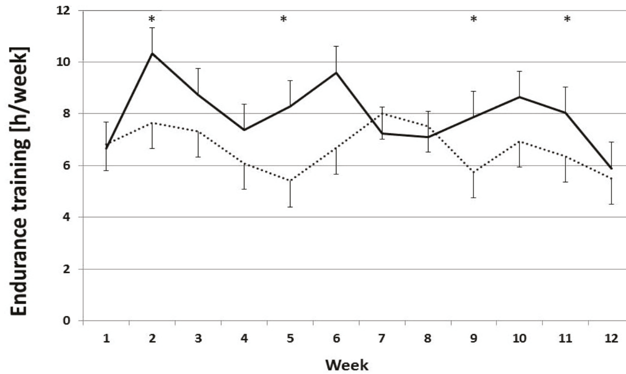

Figure 2. Training loads for endurance training (h/week) over the study period for the participants who completed the study. Graph shows mean ± standard error of the mean (SEM); * $p < 0.05$ (Mann-Whitney *U* test). Asterisks depict weeks with significant differences between PRO (—) and PLA (···) groups. PRO: probiotics-supplemented group; PLA: placebo group.

3.3. Body Composition, Nutrition, and Performance

After 12 weeks of treatment, there was no significant difference between probiotic supplementation groups and placebo groups in anthropometric characteristics, body composition, and food intake ($p > 0.05$). Performance (VO_{2max}) remained unchanged over time and still differed significantly between groups in week 12 ($p < 0.05$). Resting energy expenditure (REE, kcal/day) was significantly different between groups after 12-weeks of the study (mean ± SEM: 1617 ± 57 kcal/day and 1518 ± 56 kcal/day for PRO and PLA, respectively; $p < 0.05$, $\eta^2 = 0.13$; Figure 3).

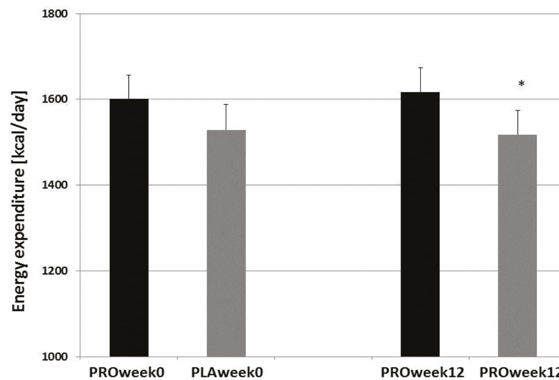

Figure 3. Resting energy expenditure (REE; (kcal/day)) in trained athletes before and after 12 weeks of treatment. PRO: probiotics-supplemented group ($n = 14$); PLA: placebo group ($n = 15$). Graph shows mean + SEM; * $p < 0.05$ (ANOVA).

3.4. Amino Acids

At the beginning of the study, exhaustive exercise induced a decrease in Trp levels in both the probiotic and the placebo group (Table 3). At the end of the experimental protocol, the exercise-induced Trp shift was comparable to the shift in week 0 in subjects who ingested probiotics but was more pronounced in in the placebo group (approximately 10% lower than in week 0, $p < 0.05$) (Figure 4).

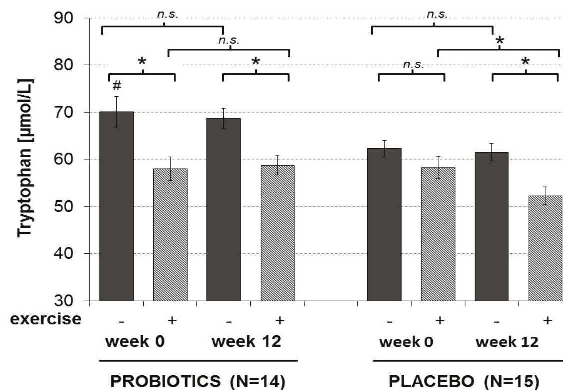

Figure 4. Tryptophan concentrations before and after exhaustive exercise in the probiotic ($n = 14$) and placebo ($n = 15$) group of trained athletes before and after 12 weeks of treatment (four blood draws per athlete). Graph shows mean ± SEM; * $p < 0.05$: Wilcoxon, # $p < 0.05$: week 0, before exercise placebo vs. probiotics: Mann-Whitney-*U*, n.s. = not statistically significant.

These data indicate reduced Trp degradation rates in subjects supplemented with probiotics, although this effect was not significant ($p = 0.13$, $\eta^2 = 0.08$). It should be mentioned that baseline Trp concentrations were slightly but significantly lower in the placebo group compared to the probiotics group, most probably due do the different percentage of female athletes in the groups. In parallel to Trp decrease, Kyn/Trp and neopterin levels were increased after exercise in both study groups at both time points.

Further, at the beginning of the study, VO_{2max} correlated significantly with baseline concentrations of Trp ($rs = 0.562$, $p = 0.001$) and this relation remained significant after 12 weeks of treatment ($r = 0.497$, $p = 0.006$) but was no longer present after intense exercise.

Tyrosine levels significantly increased and Phe/Tyr significantly decreased with exhaustive exercise ($p = 0.018$ and $p < 0.001$, respectively), but there were no significant time-dependent differences between groups. Serum concentrations of Phe were not significantly affected, either by exercise or by supplementation (Table 3).

3.5. Immune System Biomarkers

Exhausting exercise was associated with a strong increase in neopterin levels up to +61% ($U = 4.420$, $p < 0.001$) and +63% of pre-exercise values ($U = 4.660$, $p < 0.001$), before and after 12 weeks of treatment, respectively, with no significant differences between and within groups over time. However, this increase was significantly influenced by endurance training volume with a strong inverse correlation between the athletes' training status and the concentrations of neopterin at exhaustion ($rs = -0.502$, $p < 0.01$).

Kyn concentrations were slightly increased with exercise by 7% ($U = 2.671$, $p < 0.01$) before and by 3% ($U = 0.923$, n.s.) after 12 weeks of intervention, contributing to the elevation of the Kyn/Trp ratios by 22% ($U = 4.544$, $p < 0.001$) and by 21% ($U = 4.433$, $p < 0.001$), respectively. Exercise induced a change in Kyn levels with time (ΔKyn), with a significant decline being overserved in the PLA

group (p = 0.04), whereas an increase was seen in the PRO group, but this effect was not significant between groups (p = 0.05, η^2 = 0.13). At baseline, neopterin and Kyn/Trp ratios correlated significantly (rs = 0.490, p < 0.01), with the association even becoming slightly stronger upon exercise (rs = 0.512, p < 0.01). After 12 weeks there was no longer a significant relationship between pre-exercise neopterin and Kyn/Trp levels (rs = 0.280, n.s.), but it became again significant after exercise (rs = 0.583, p = 0.001). At the same time, higher neopterin levels correlated with lower Trp concentrations (rs = −0.384, p < 0.05).

Table 3. Amino acids and immune biomarkers in 29 athletes before and after 12 weeks of treatment either supplemented with probiotics or placebo measured before (PRE) and after exercise (POST).

Probiotics (*n* = 14)	**Baseline PRE**	**Baseline POST**	**Week 12 PRE**	**Week 12 POST**
Tryptophan (µmol/L)	70.07 ± 3.20 [a,e]	57.99 ± 2.47 [b]	68.64 ± 2.12 [c,k]	58.76 ± 2.11 [d]
Kynurenine (µmol/L)	1.98 ± 0.11	1.97 ± 0.07	1.83 ± 0.10	1.92 ± 0.11
Kyn/Trp (µmol/mmol)	28.35 ± 1.16 [f]	34.50 ± 1.46	26.94 ± 1.51 [l]	33.32 ± 2.19
Neopterin (nmol/L)	5.19 ± 0.23 [g]	8.43 ± 1.00	4.92 ± 0.31 [m]	7.74 ± 0.86
Tyrosine (µmol/L)	138.58 ± 29.96 [h]	145.06 ± 6.23	147.25 ± 24.01 [n]	149.22 ± 5.60
Phenylalanine (µmol/L)	69.59 ± 8.27 [i]	68.72 ± 2.06	72.16 ± 1.99 [o]	71.76 ± 1.90
Phe/Tyr (mol/mol)	0.52 ± 0.08 [j]	0.48 ± 0.01	0.50 ± 0.03 [p]	0.49 ± 0.02
Placebo (*n* = 15)	**Baseline PRE**	**Baseline POST**	**Week 12 PRE**	**Week 12 POST**
Tryptophan (µmol/L)	62.27 ± 1.72	58.27 ± 2.37	61.50 ± 1.84	52.26 ± 1.86
Kynurenine (µmol/L)	1.77 ± 0.13	2.02 ± 0.07	1.75 ± 0.08	1.77 ± 0.09
Kyn/Trp (µmol/mmol)	28.38 ± 1.81	34.49 ± 2.17	28.49 ± 1.03	34.03 ± 1.51
Neopterin (nmol/L)	6.63 ± 0.95	10.48 ± 1.56	5.65 ± 0.70	9.55 ± 2.06
Tyrosine (µmol/L)	131.15 ± 5.28	137.40 ± 5.42	126.41 ± 6.29	129.28 ± 5.76
Phenylalanine (µmol/L)	69.23 ± 2.45	68.55 ± 1.87	72.53 ± 1.67	70.09 ± 2.71
Phe/Tyr (mol/mol)	0.53 ± 0.02	0.50 ± 0.02	0.59 ± 0.03	0.55 ± 0.02

Values are means ± SEM. [a] U = 2.095, p = 0.036 (baseline PRE placebo vs. probiotics), [b] U = 0.284, p = 0.777 (baseline POST placebo vs. probiotics), [c] U = 2.706, p = 0.007 (week 12 PRE placebo vs. probiotics), [d] U = 2.139, p = 0.032 (week 12 POST placebo vs. probiotics), [e] U = 3.384, p = 0.001 (all athletes baseline PRE vs. POST), [f] U = 4.660, p < 0.001 (all athletes baseline PRE vs. POST), [g] U = 4.420, p < 0.001 (all athletes baseline PRE vs. POST), [h] U = 2.011, p = 0.044 (all athletes week 12 PRE vs. POST), [i] U = 0.270, p = 0.787 (all athletes week 12 PRE vs. post), [j] U = 3.357, p = 0.001 (all athletes week 12 PRE vs. post), [k] U = 4.703, p < 0.001 (all athletes week 12 PRE vs. POST), [l] U = 4.433, p < 0.001 (all athletes week 12 PRE vs. post), [m] U = 4.544, p < 0.001 (all athletes week 12 PRE vs. post), [n] U = 0.443, p = 0.658 (all athletes week 12 PRE vs. post), [o] U = 0.660, p = 0.510 (all athletes week 12 PRE vs. post), [p] U = 1.208, p = 0.227 (all athletes week 12 PRE vs. post).

3.6. Infection Incidence

Only one participant on the placebo experienced GI-discomfort symptoms during the study period. Analysis of the URTI-symptom questionnaires indicated that 55% (16 subjects) of the cohort experienced an URTI episode during the 12-week study period. Thirteen subjects did not experience any URTI episode during the study period. Before supplementation, 10 subjects on placebo and 12 subjects on probiotics experienced one or more URTI symptoms over the prior three months. After 12 weeks of treatment, 11 subjects on placebo and 5 subjects on probiotics experienced one or more URTI symptoms during the study period (Figure 5). The proportion of subjects who experienced one or more URTI symptoms during the study period was 2.2-fold higher in the placebo group than in the probiotics group (PLA 0.79, PRO 0.35; p = 0.016).

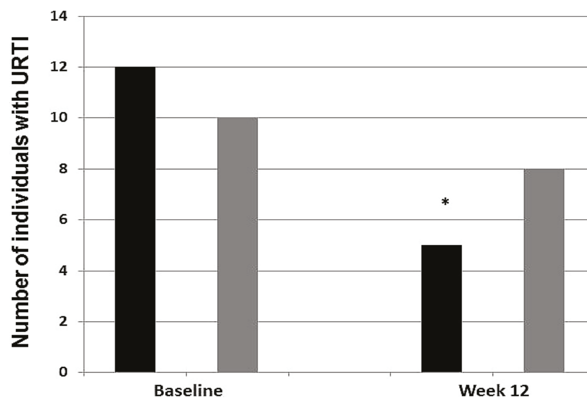

Figure 5. Incidence of upper respiratory tract infections (URTIs) in trained athletes before and after 12 weeks of treatment. The share of subjects on placebo (gray columns, 0.79) who experienced 1 or more URTI symptoms was 2.2-fold greater than those on probiotics (black columns, 0.35; * p = 0.016).

Individuals who developed URTI had higher degradation rates of Trp before exercise compared to those without URTI (Table 4). Additionally, a running nose, but not cough was associated with higher Kyn/Trp ratios compared to those individuals without such symptoms.

Table 4. Association between upper respiratory tract infection (URTI) incidence at week 12 and degree of tryptophan breakdown as indicated by Kyn/Trp (mean ± SEM). Bold text indicates a statistically significant correlation with a p-value less than 0.05.

URTI	Baseline PRE	Baseline POST	Week 12 PRE	Week 12 POST
yes	28.9 ± 1.7	34.9 ± 6.0	31.1 ± 5.1	38.9 ± 7.3
no	28.2 ± 6.5	34.4 ± 7.2	26.7 ± 4.3	32.0 ± 6.0
U/p-value	0.535/0.592	0.102/0.919	**2.039/0.041**	**2.090/0.037**

4. Discussion

This study illustrates a significant influence of probiotic supplementation on athletes who performed intense exercise. On the one hand, increased training load was measured and on the other hand, the rate of infectious complications was markedly reduced. However, whether this is based on the actual probiotic supplementation or due to other cofounding factors (baseline fitness, gender) is currently unknown. In addition, some of these influences appeared to be connected with alterations in Trp metabolism, e.g., Trp breakdown rates at the end of the study were significantly higher in individuals who developed infections as compared to those who did not. However, it was not determined whether higher Kyn/Trp ratios were observed, particularly in those individuals who experienced an infection close to the end of the study and it still remains to be elucidated whether there is a more direct association between probiotic supplementation and reduced Trp breakdown. Alternatively, different training loads between groups may have affected Trp metabolism, rather than the actions of the probiotic, since regular endurance exercise causes adaptations in Kyn metabolism [41].

4.1. Training Adherence

Supplementation with probiotics was associated with higher training loads vs. placebo. One explanation for these findings could be that probiotics may enable better performance capabilities and training adherence when the risk of URTI development is reduced, as individuals with fewer episodes of infections such as common colds and runny noses are able to train more often and harder

than others. However, it could also be possible that existing URTI symptoms influenced training performance to a lesser extent in athletes on probiotics [31]. In any case, performance was not increased even with the higher training load in the probiotics group as compared to the placebo group, even if the training load was indeed an effect of the supplementation.

A potential role of Trp metabolism could be of relevance for the effects of probiotics on training adherence because individuals on probiotics showed higher serum Trp levels than those without such supplements. Higher serum Trp levels may improve the Trp transport into brain and support serotonin metabolism, which can influence an individual's sensation of fatigue and thus potentially affect training adherence and performance [42]. Interestingly, VO_{2max} correlated with pre-exercise Trp levels supporting a role of Trp metabolism in training performance. It could further relate to the recent findings of Kyn metabolism in skeletal muscle mediating resilience to stress-induced depression with endurance training, whereas less energetically demanding exercise protocols, such as high-force eccentric exercise, did not lead to adaptations in Kyn metabolism [41,43].

4.2. Tryptophan and the Gut Microbiome

Post exercise serum Trp levels declined but this was only true in the placebo group whereas serum Trp levels did not change but remained stable in individuals supplemented with probiotics. This difference could be due to an effect of probiotics on the microbiome composition in the gut, which may affect downstream immunoregulatory pathways. Alterations in the gut milieu influence Trp metabolism and the absorption and availability of the essential amino acids [17]. In addition, the altered composition of the microbiome may increase the biosynthesis of Trp by specific bacteria. Research in rats has shown that administration of the probiotic *Bifidobacteria infantis* attenuated pro-inflammatory immune responses following mitogen stimulation and, furthermore, there was a marked increase in plasma concentrations of Trp in the *Bifidobacteria*-treated rats when compared to controls [44], suggesting that bacteria can improve the available serotonin pool and ultimately elicit communication between the gut and the brain via serotonin [17,18]. In the present study, probiotics were able to selectively modulate Trp concentrations since no influence on the metabolism of Phe, another essential amino acid, was observed. Interestingly, no detectable effect of supplementation was found on concentrations of immune system biomarker neopterin and also Kyn/Trp ratios were not modulated. Further studies will be necessary to address these open questions.

4.3. Probiotics to Prevent URTIs

Some well-controlled studies in athletes have shown that daily probiotic ingestion results in fewer days of respiratory illness and lower severity of URTI symptoms [30–32]. A meta-analysis using data from both athlete and non-athlete studies concluded that there is a likely benefit of reducing URTI incidence [45]. The likely mechanisms of action for probiotics include direct interaction with the gut microbiota, interaction with the mucosal immune system and immune signaling to a variety of organs and systems [46]. A recent report by He and colleagues noted gender differences in the number and duration of respiratory-tract illness symptoms in endurance athletes during a winter training period indicating that females may be more susceptible to URTI than their male counterparts [5]. Furthermore, supplementation with *Lactobacillus fermentum* was associated with a reduction of the symptoms in clinical indices of URTI at high training loads in well-trained male cyclists but not in females, for whom there was some evidence of an increase in symptoms [47]. Thus, females may benefit from higher doses of probiotics. In the present study, females were overrepresented in the control group who experienced more than double the URTI symptoms, accompanied by higher Trp breakdown rates compared to those on probiotics. However, gender is not the basis for the observed effect on URTI incidence in this study. The interaction between gender and URTI was not statistically significant in either group before and after 12 weeks of treatment (Table 5). It remains unclear whether these findings are related to the influence of gender on Trp catabolism and further work is required to address this issue.

Table 5. Interaction between gender and illness symptoms in 29 athletes before and after 12 weeks of treatment either supplemented with probiotics (PRO) or placebo (PLA).

Illness Symptoms		Baseline U	p	Week 12 U	p
URTI	PRO	0.212	0.832	0.155	0.877
	PLA	0.748	0.454	0.399	0.690
Runny nose	PRO	0.212	0.832	0.329	0.742
	PLA	0	1	1.080	0.280
Cough	PRO	0.931	0.352	0.362	0.717
	PLA	1.497	0.134	1.497	0.134
Sore throat	PRO	1.041	0.298	0.866	0.386
	PLA	0.374	0.708	1.497	0.134
Fever	PRO	0.823	0.411	0	1
	PLA	0	1	0	1
Weakness	PRO	0.866	0.386	0	1
	PLA	0.519	0.604	0.519	0.604

Taken together, probiotic supplements on a daily basis enhance resistance to URTI in athletes and offer thus a potential intervention strategy during heavy exercise training periods, especially in the winter months. A prerequisite for robust immune function during intense exercise is, however, to avoid a long-term energy deficit, deficiencies of macronutrients and essential micronutrients, and to ingest carbohydrate during exercise [48].

4.4. Study Strengths and Limitations

This study has several strengths and limitations. A major strength was the randomized controlled, double-blinded, placebo-controlled study design and the use of an objective and standardized test for assessment of peak oxygen uptake and peak power output. The study was conducted with a multi-species probiotic consisting of *Bifidobacterium bifidum* W23, *Bifidobacterium lactis* W51, *Enterococcus faecium* W54, *Lactobacillus acidophilus* W22, *Lactobacillus brevis* W63, and *Lactococcus lactis* W58 at a total dose of 1 billion CFU. Total probiotic cell count varied between 11×10^9 CFU/g at the beginning of the study and 7×10^9 CFU/g at the end of the study. The individual amount of each strain is unknown. Returned sachet count after the treatment period revealed a high level of compliance in both groups (>95%). The 80% cutoff has been used for a majority of studies on medication adherence, especially for cardiovascular medications, since adherence based on this cutoff point has been associated with both intermediate and strong outcomes [49]. Although the 80% cutoff appears reasonable, the optimal level of adherence for dietary supplements may be higher than current cutoffs (e.g., 80% to 100%). Limitations of the study are the relatively small sample size and significant differences in gender composition of the subpopulations, which may have contributed to differences in illness symptoms and physiological parameters, e.g., VO_{2max} and weekly training logs between males and females. We did not randomize by body weight, performance, or any other variable that might have given us a better chance to catch differences in outcomes since women—overrepresented in the control group—generally show a lower VO_{2max}. Indeed, West et al. (2011) showed gender difference with probiotic supplementation in athletes with a significant reduction in respiratory infections (duration and severity) in males, but no effect in females [47]. Therefore, we analyzed the influence of gender on URTI. No significant effect of gender was observed for either group, before or after 12 weeks of treatment. However, Trp metabolism can be influenced to some extend by gender differences [50]. This aspect is mainly relevant for the results obtained at the baseline when Kyn levels were found to differ between groups. Another limitation of the study is that we were not able to calculate the severity of illness symptoms because of the high number of no replies. Furthermore, infections were only symptomatically monitored, but not serologically proven. It is assumed that

common pathogens either of bacterial or viral origin have been involved in and could contribute to alterations of, e.g., Trp metabolism, which, because of its immunoregulatory influence, could increase the risk of such infections. Longitudinal research will be needed to clarify causal ordering.

5. Conclusions and Future Research

Daily supplementation with probiotics was found to be associated with a lower frequency of URTIs in athletes who underwent endurance training and seems to be beneficial in increasing training efficacy during training periods, however, no benefits to athletic performance were observed. Some of these effects appeared to be connected with alterations in Trp metabolism. Still, these findings are of a preliminary nature and warrant further investigation into the precise mechanisms involved. In addition, more research is required to clarify issues of strains, dose–response, mechanisms and best practice models for probiotic implementation in various sports disciplines. It should be further investigated as to whether regular exercise per se affects human microbiota characteristics, for how long and how much exercise is needed.

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3

Nutrition Assessment of B-Vitamins in Highly Active and Sedentary Women

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Abstract: Background: Female athletes and active women require adequate nutrition for optimal health and performance. Nutrition assessments are needed to identify potential nutrients of concern. Folate, vitamin B6, and vitamin B12 function in important pathways used during physical activity and female athletes may be at risk for poor status of these micronutrients. This cross-sectional study described a comprehensive nutrition assessment of the B-vitamins (folate, vitamin B6, and vitamin B12) using both dietary (food and dietary supplements) and biochemical assessments among highly active and sedentary women. Methods: Highly active (n = 29; age 20 ± 2 years; body mass index (BMI) 23.8 ± 3.5 kg/m^2) and sedentary (n = 29; age 24 ± 3 years; BMI 22.6 ± 3.0 kg/m^2) women were recruited for this study. Participants completed 7-day weighed food records and a fasting blood draw. Results: Although the highly active women reported higher intakes of energy ($p < 0.01$), folate ($p < 0.01$), vitamin B6 ($p < 0.01$), and vitamin B12 ($p < 0.01$), no significant differences were found between the groups for biomarkers of folate, vitamin B6, and vitamin B12. All of the highly active women had biomarkers within the desired reference ranges, suggesting good status. In general, most participants were able to meet the 1998 Recommended Daily Allowance (RDA) from food alone. For the women that reported using dietary supplements, micronutrient intakes met the 1998 RDA and in some cases, exceeded the Tolerable Upper Intake Level. Conclusion: This nutrition assessment documented good status for folate, vitamin B6, and vitamin B12 in the highly active women. Similar assessment approaches (food, dietary supplements, and biomarkers) should to completed with other nutrients of concern for the female athlete.

Keywords: B-vitamins; folate; vitamin B6; vitamin B12; female athlete

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1. Introduction

Female athletes and active women require adequate nutrition to stay healthy and perform optimally. Comprehensive nutrition assessments, including dietary, biochemical, anthropometric, clinical, and environmental components, are needed to identify specific nutrition-related problems that may impact overall health and performance. For example, the B-vitamins play important roles in maintaining the health of female athletes and active women, serving as coenzymes in pathways critical for physical activity [1–6]. Folate functions as a coenzyme in reactions of deoxyribonucleic

acid (DNA) synthesis, red blood cell synthesis, and amino acid metabolism, including the conversion of homocysteine to methionine [4,7]. Pyridoxal 5′-phosphate (PLP), the biologically active form of vitamin B6 in the human body, serves as a coenzyme in transamination and deamination reactions of amino acid metabolism and activates the rate-limiting step of glycogen breakdown [4,8,9]. Vitamin B12 functions as a coenzyme in methyl transfer reactions (i.e., homocysteine to methionine) and helps recycle folate [4,10]. Vitamin B12 also assists with the breakdown of odd-numbered fatty acid chains, DNA synthesis, and the production of red blood cells. Because folate, vitamin B6, and vitamin B12 assist with the metabolism of homocysteine, plasma homocysteine concentrations increase without adequate supplies of folate, vitamin B6, and vitamin B12, leading to an increased risk of cardiovascular disease [11]. Thus, these B-vitamins aid in the utilization of energy, metabolism of amino acids, maintenance of red blood cells, and regeneration of tissue. Comprehensive nutrition assessment of these key micronutrients is crucial to an athlete's success.

Unfortunately, many athletes, females in particular, may be at risk of poor dietary intakes for folate, vitamin B6, and vitamin B12 [12]. For instance, female athletes may not compensate for the energy expenditure associated with increased physical activity [13]. This behavior puts them at risk of low energy availability and many macronutrient and micronutrient deficits. In many sports, success is associated with a thin physique, thus encouraging excessive training and/or suboptimal dietary intakes [14,15]. Unfortunately, inadequate dietary intakes can impair an athlete's performance and lead to fatigue, injury, and/or altered concentration [12]. Additionally, female athletes and active women may have less folate, vitamin B6, and vitamin B12 available for metabolism of homocysteine, potentially leading to elevated plasma homocysteine concentrations compared to their physically inactive peers.

Dietary assessment has been utilized to assess dietary intakes for folate, vitamin B6, and vitamin B12 and determine adequacy. Recent dietary intakes for the United States (US) adult population have been well summarized from the National Health and Examination Surveys (NHANES) [16]. For female athletes and active women, reported dietary intakes for these micronutrients tend to come from older studies and are challenging to interpret due to changes in the reference ranges used to define nutrient adequacy. When completing dietary assessment for folate, time of data collection should also be considered to account for the 1996 US mandatory fortification of enriched grain products with folic acid [17]. In 1998, the latest reference values for these micronutrients were published as part of the Dietary Reference Intakes (DRIs) [18]. The DRIs express nutrient adequacy as the Estimated Average Requirement (EAR) (representing 50% of the population's requirement) and the Recommended Dietary Allowance (RDA) (representing 97.5% of the population's requirement). However, the EAR may not be sufficient for a physically active adult, adding to the difficulty to make generalizations about nutrient adequacy. Notwithstanding these concerns, some studies report mean dietary intakes for folate in female athletes less than the 1998 EAR (320 μg/day DFE) [19–24]. However, more recent studies report higher dietary intakes for folate among female athletes [25,26], which may be a reflection of folic acid fortification. For vitamin B6, most studies in female athletes document adequate intakes when compared to the 1998 RDA (1.3 mg/day) and EAR (1.1 mg/day) [21,22,25,26]. Studies comparing dietary intakes of vitamin B6 to the 1980 RDA (2 mg/day for adult females) or 1989 RDA (1.6 mg/day for adult females), values much higher than the 1998 RDA, typically report inadequate mean intakes for female athletes [4,19,22,27]. Because vitamin B6 plays a major role in the metabolic pathways required during exercise (i.e., amino acid metabolism, gluconeogenesis, glycogenolysis), some research suggests that female athletes may require two to three times the 1998 RDA of vitamin B6 due to their increased physical activity patterns and protein requirements [4,8]. For vitamin B12, some studies report adequate dietary intakes in female athletes [19–21,25,26], while other studies report inadequate intakes [13,23].

Because of the risk of inadequate dietary intakes of folate, vitamin B6, and vitamin B12 in female athletes, some research has included biochemical assessment in the evaluation of nutrient status. Unfortunately, the results are quite mixed. For example, Matter et al. examined folate status in

non-supplementing female marathon runners and reported that 33% had poor status evidenced by low serum folate concentrations [28]. However, Beals and Manore examined serum folate concentrations in female athletes and reported only 4% with poor folate status [29]. Approximately 50% of the female athletes in this study reported taking a dietary supplement. Other research has reported good folate status in female recreational athletes [26,30], runners [31], and endurance athletes [32]. Research has also examined vitamin B6 status in both male and female athletes, with equally mixed outcomes. For instance, Raczynski and Szczepanska assessed vitamin B6 status of elite male and female Polish athletes over 6 years using the erythrocyte alanine aminotransaminase activity coefficient, a functional measure of vitamin B6, and reported poor status in 9% of the athletes [33]. In this study, endurance athletes had the highest prevalence of poor status of vitamin B6 (13%). Poor vitamin B6 status was highest in the pre-Olympic years (16%) and lowest in Olympic years (3%), when athletes may have focused more on dietary intakes and dietary supplementation. More recently, Joubert and Manore reported good status in a study of 64 recreationally active athletes (38 female) using plasma PLP [26]. Most studies of vitamin B12 status in female athletes suggest the risk of poor status is low, when adequate energy and animal products are consumed. Although the research is much more limited, good vitamin B12 status has been reported in female ultra-marathoners [34] and recreationally active adults [26].

More research should include comprehensive nutrition assessments to examine B-vitamin status in female athletes, especially for folate, vitamin B6, and vitamin B12. When completing dietary assessments, previous research has not included the contribution of natural sources and synthetic sources (fortified foods, dietary supplements) to dietary intakes. To determine nutrient status, biochemical assessment should also be included. Unfortunately, mixed gender studies have included more male participants than female participants, limiting information on the B-vitamin status of the female athlete. Thus, the purpose of this study was to describe the approach and results of a comprehensive nutrition assessment for B-vitamins (folate, vitamin B6, and vitamin B12), including dietary (food and dietary supplements) and biochemical assessments, among highly active and sedentary women.

2. Methods

2.1. Participant Recruitment and Study Design

This cross-sectional study completed a nutrition assessment of the B-vitamins (folate, vitamin B6, and vitamin B12) among highly active and sedentary women. This study was approved by the Institutional Review Board (IRB) at Arizona State University (IRB #0511000343; initial approval date 15 December 2005) and the University Committee on Activities Involving Human Subjects at New York University (IRB #11-8778; initial approval date 9 January 2012) and was conducted according to these guidelines.

Highly active and sedentary women between 18 and 35 years of age were recruited as the research participants for this study. Recruitment flyers were posted at university and college campuses, athletic training facilities, community centers, libraries, and throughout the local community. The study investigators also sent recruitment flyers to collegiate teams. The recruitment flyers briefly described the study and invited women to contact the study investigators for more information.

The study investigators determined eligibility over the telephone based on the following criteria: age (between 18 and 35 years), weight stable (<10% weight loss or gain within the past 6 months), no pregnancy or breastfeeding within the past year, nonsmoker or limited social smoker (quit smoking at least 6 months prior to study entry or smoke a few cigarettes socially on one occasion and then not smoke again for several days or weeks), and activity (highly active group defined as engaging in $\geq$12 h per week of programmed physical activity; sedentary group defined as engaging in <2 h of programmed physical activity per week). These activity levels must have been maintained for at

least a year prior to study participation. Women who met the study criteria were invited to schedule a study appointment.

2.2. Procedures

The study participants completed two study visits. During the first visit, participants received detailed information about the study and signed an informed consent form. Height and weight were measured and body mass index (BMI) was calculated for each study participant. Participants were interviewed about the use of medications (prescription and over-the-counter) and dietary supplements (i.e., protein, energy, carbohydrate, meal replacement, vitamin, mineral, or herbal) and completed a health history questionnaire. Participants were asked to keep a 7-day weighed food record, noting all foods, beverages, and dietary supplements consumed. The study investigators provided participants with a food scale (Metrokane Gourmet Weigh Scale, Metrokane, New York, NY, USA) and showed them how to weigh foods. Participants were encouraged to include food labels for packaged items and provide measurements in teaspoons, tablespoons, or cups for foods not able to be weighed.

A second study visit was scheduled after the participants completed the food records. At this visit, participants completed an eight-hour fasting blood draw to determine blood biomarkers of folate, vitamin B6, and vitamin B12. The food records and study questionnaires were reviewed for completeness and study supplies were retrieved.

2.3. Anthropometric Assessment

Height was determined using a portable stadiometer (Invicta Plastics Limited, Oadby, Leicester, UK) to the nearest 0.1 centimeter without shoes. A Seca Bella 840 electronic flat scale (Seca North America, Chino, CA, USA) obtained each participant's weight to the nearest 0.1 kilogram.

2.4. Dietary and Physical Activity Assessment

Participants completed a 7-day weighed food record to examine dietary intake of energy, folate, vitamin B6, and vitamin B12. At the end of each day, participants recorded the type and duration of any programmed physical activity completed. The food records were analyzed using Food Processor, version 8.5 (Esha Research, Salem, OR, USA) and the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 20. The USDA database provided additional micronutrient information for commercial products. Folate intakes included assessments of dietary food folate (natural), synthetic folic acid added to fortified foods, and dietary folate equivalents (DFE) ((synthetic folic acid $\times$ 1.7) + food folate (natural)). The intake total included both natural and synthetic forms of vitamin B12. For vitamin B6, the intake total included vitamin B6 from food. Dietary intakes over the 7-days were averaged to determine the reported daily intake.

Micronutrient intakes (folic acid, vitamin B6, and vitamin B12) from supplements were added to the totals from food for those participants that reported using dietary supplements. The amount of folic acid from supplements was multiplied by 1.7 before adding to the total from food. Intakes from dietary supplements were summarized to reflect reported average daily intakes, considering dosage and usage patterns (days/week or days/month).

The estimated energy requirement (EER) was calculated for each participant using the appropriate age and gender equation [35]. For the active women, the "very active" physical activity coefficient was used in the equation. For the sedentary women, the "sedentary" physical activity coefficient was used. Energy intake/EER was determined for each participant.

2.5. Biochemical Assessment

Participants completed an eight-hour fasting blood draw to determine concentrations of plasma folate, red blood cell folate, plasma vitamin B6, vitamin B12, transcobalamin II, and homocysteine. Blood samples were immediately placed on ice and centrifuged within 30 min at $3000\times g$ for 10 min at 4 °C. After centrifugation, the blood was separated and the plasma samples stored at −44 °C until

analysis. Sonora Quest (Phoenix, AZ, USA), an independent laboratory, determined mean cell volume, hemoglobin, hematocrit, and high sensitivity C-reactive protein (CRP) concentrations.

For the analysis of red blood cell folate, a whole blood dilution (1:21) was prepared by combining 100 µL of well-suspended blood to 2 mL of newly made 0.2% ascorbic acid solution. The diluted samples were wrapped in foil to prevent light penetration and stored at −44 °C until the time of analysis. Plasma folate and red blood cell concentrations were analyzed using the Becton Dickinson SimulTRAC®-S Solid Phase Radioassay Kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for Vitamin B12 (^{57}Co) and Folate (^{125}I).

High performance liquid chromatography (HPLC) was used to determine vitamin B6 status of the participants using PLP as the biomarker [36]. HPLC utilizes reverse-phase ion pairing to separate B6 vitamers, which are chromatographically measured at an excitation wavelength of 330 nm and fluorescent emission of 400 nm.

Vitamin B12 status was assessed using plasma vitamin B12 and holotranscobalamin II (transcobalamin II) concentrations [37]. Transcobalamin II represents newly absorbed vitamin B12 enroute to the hematopoietic system and proliferating cells and is a more sensitive indicator of vitamin B12 status than plasma vitamin B12. Transcobalamin II was assessed by first preparing a slurry that contained 3 g synthetic amorphous precipitated silica in 20 mL of deionized water [38,39]. Transcobalamin II was absorbed from the samples by adding 100 µL of the prepared slurry to 500 µL of plasma and letting the samples sit at room temperature for 10 min. The samples were then centrifuged at 5000× g for 10 min. The supernatant was retained for further analysis. The Becton Dickinson SimulTRAC®-S Solid Phase Radioassay Kit (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) for Vitamin B12 (^{57}Co) and Folate (^{125}I) was used to measure plasma vitamin B12 and holo-haptocorrin concentrations. Transcolbalamin II concentrations were determined by subtracting the holo-haptocorrin concentrations from the total plasma vitamin B12 concentration.

Fasting plasma homocysteine, a functional biomarker of folate, vitamin B6, and vitamin B12 status, was also measured by HPLC with fluorescence [40,41].

2.6. Statistical Analysis

Power calculations were completed using the reported vitamin B6 and folate dietary intakes for athletes and sedentary individuals from the research literature [42,43]. Using a difference of 0.2 mg, a sample size of 15 women per group would be sufficient to detect a difference in reported vitamin B6 intake between groups with a power of 0.80 and $\alpha = 0.05$. However, a sample size of 57 women per group would be required to detect a difference in reported folate intake (using a difference of 50 µg) between groups with a power of 0.80 and $\alpha = 0.05$, beyond the reach of this pilot study. Thus, we aimed to have 30 women per group and recruited additional women to allow for attrition. Prior to the statistical analysis, the data were tested for normality. Histograms of the study outcome measures visually assessed the distribution of the data. The Kolmogorov-Smirnov statistic was also used to assess normality of the distribution scores. Descriptive statistics (mean and standard deviation) were determined for the demographic data for the two groups of women. Independent sample t-tests compared the outcome measures between groups for the normally distributed variables. Because CRP did not have a normal distribution, the Mann-Whitney U test was used to examine the differences between groups. The median and interquartile range were used to summarize these values. Data were analyzed using IBM Statistical Package for the Social Sciences (SPSS) Statistics for Windows, version 22.0 (IBM Corporation, Armonk, NY, USA) version 14.0 and determined to be significant if $p < 0.05$.

3. Results

3.1. Descriptive Characteristics

Seventy-five participants signed consent forms (41 highly active women and 34 sedentary women). However, 11 highly active and 3 sedentary women decided not to finish the study. One highly active

woman was eliminated because she was not as active as previously reported. Two sedentary women were eliminated; one had a blood disorder, and one had an activity level that was greater than 2 h per day. The present analysis includes 29 highly active women and 29 sedentary women.

Table 1 summarizes the descriptive characteristics for the 58 participants. The sedentary women were older than the highly active women ($p < 0.01$). Although the highly active women were heavier than the sedentary women, height and BMI were not significantly different between the two groups of women. The majority of participants in both groups, 69% of the highly active women and 82% percent of the sedentary women, reported their race/ethnicity as Caucasian (not of Hispanic origin). The highly active women reported consuming more total energy ($p < 0.01$) and relative energy (kcal/kg body weight) ($p = 0.01$) than the sedentary women. Although EER was significantly greater in the highly active women compared to the sedentary women ($p < 0.01$), there were no differences between groups for percent energy intake/EER.

Table 1. Descriptive characteristics of highly active and sedentary women [a].

Characteristics	Activity Level		*p*-Value
	Highly Active	Sedentary	
	n = 29	*n* = 29	
Descriptives			
Age (years)	20 ± 2	24 ± 3	<0.01 **
Height (cm)	169 ± 7	166 ± 8	0.11
Weight (kg)	68 ± 9	62 ± 10	0.03 *
Body mass index (kg/m^2)	23.8 ± 3.5	22.6 ± 3.0	0.17
Programmed physical activity (min/day)	169 ± 241	6 ± 8	
Energy intake			
Total energy (kcal/day)	2373 ± 616	1820 ± 403	<0.01 **
Relative energy (kcal/kg body weight)	35.2 ± 8.9	29.6 ± 7.2	0.01 *
Energy expenditure			
Estimated energy requirement (EER) (kcal)	2350 ± 168	1972 ± 132	<0.01 **
Energy intake/EER (%)	101 ± 25	92 ± 19	0.15
Race/ethnicity [b]			
African American (*n* (%))	0 (0)	0 (0)	
Asian/Pacific Islander (*n* (%))	1 (3)	0 (0)	
Native American (*n* (%))	0 (0)	1 (4)	
Caucasian (not of Hispanic origin) (*n* (%))	20 (69)	23 (82)	
Hispanic (*n* (%))	8 (28)	4 (14)	
Sport			
Basketball (*n* (%))	1 (3)		
Cross country/Long distance running (*n* (%))	3 (10)		
Gymnastics (*n* (%))	1 (3)		
Ice hockey (*n* (%))	1 (3)		
Softball (*n* (%))	3 (10)		
Swimming (*n* (%))	11 (38)		
Tennis (*n* (%))	4 (11)		
Volleyball (*n* (%))	5 (17)		

[a] Values are reported as mean ± standard deviation, except where noted; [b] One sedentary participant did not provide this information; * $p < 0.05$; ** $p < 0.01$.

Table 1 also describes the highly active women by their sport. The highly active women consisted of student athletes from Division I, Division II, and community college athletic teams.

3.2. Dietary Assessment

Tables 2–6 summarize the reported micronutrient intake of the 58 highly active and sedentary women. Folate intakes from food (natural sources, fortified foods) and dietary supplements are summarized in Table 2. The highly active women reported a greater intake of natural folate (μg/day) ($p < 0.01$), folic acid from fortified foods (μg/day) ($p = 0.03$), and folate (natural + fortified foods) (μg/day dietary folate equivalents (DFE)) ($p < 0.01$) than the sedentary women. There were no differences between the two groups of women for folate density (μg/day DFE/1000 kcal). For those participants that reported dietary supplement use (highly active = 9; sedentary = 12), the sedentary women consumed more folic acid from dietary supplements (μg/day) ($p = 0.04$) than the active women. However, there were no additional differences in folate intakes between the groups for participants that reported using dietary supplements.

Table 2. Folate intakes (food and dietary supplements) in highly active and sedentary women [a].

Intake Variable [b]	Activity Level		*p*-Value
	Highly Active	Sedentary	
	n = 29	*n* = 29	
Folate intake from food			
Folate (natural) (μg/day)	284 ± 119 256 (175)	190 ± 75 190 (116)	<0.01 **
Folic acid (fortified foods) (μg/day)	345 ± 213 302 (272)	238 ± 140 197 (155)	0.03 *
Folate (natural + fortified foods) (μg/day DFE) [c,d]	867 ± 391 777 (520)	595 ± 250 537 (344)	<0.01 **
Folate density (μg DFE/1000 kcal) [c,e]	364 ± 135 325 (205)	336 ± 154 288 (132)	0.47
Supplement contribution for those that reported supplement use			
Participants that reported folate dietary supplement use (*n* (%))	9 (31)	12 (41)	
Folic acid (dietary supplements) (μg/day)	564 ± 272 588 (364)	935 ± 438 680 (595)	0.04 *
Folate (natural) + folic acid (fortified foods + dietary supplements) (μg/day DFE) [c,d]	1470 ± 672 1232 (941)	1468 ± 473 1447 (670)	0.99
Folate (natural) + folic acid (fortified foods + dietary supplements) density (μg DFE/1000 kcal) [c,e]	621 ± 299 509 (358)	904 ± 365 775 (508)	0.07

[a] Values expressed as mean ± standard deviation and median (interquartile range), except where noted. [b] Intake variable determined by 7-day weighed food records analyzed using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 20. [c] μg/day of DFE (Dietary Folate Equivalents) = (folic acid × 1.7) + natural food folate. [d] Folate Recommended Dietary Allowance (RDA) for women ages 14–18 = 400 μg/day of DFE. Folate RDA for women ages 19–50 = 400 μg/day of DFE. [e] Recommended value for DFE density: 250 μg of DFE/1000 kcals [44]. * $p < 0.05$. ** $p < 0.01$.

Table 3. Vitamin B6 intakes (food and dietary supplements) in highly active and sedentary women [a].

Intake Variable [b]	**Activity Level**		***p*-Value**
	Highly Active ***n* = 29**	**Sedentary** ***n* = 29**	
Vitamin B6 intake from food			
Vitamin B6 (mg/day) [c]	3.5 ± 2.2 2.8 (1.9)	1.8 ± 0.7 1.6 (0.8)	<0.01 **
Vitamin B6 density (mg/1000 kcal) [d]	1.6 ± 1.2 1.1 (0.8)	1.0 ± 0.4 0.9 (0.5)	0.03 *
Supplement contribution for those that reported supplement use			
Participants that reported vitamin B6 dietary supplement use (*n* (%))	8 (28)	12 (41)	
Vitamin B6 (dietary supplements) (mg/day)	7.6 ± 9.2 2.7 (14.0)	14.0 ± 29.4 2.3 (3.0)	0.56
Vitamin B6 (food) + vitamin B6 (dietary supplements) (mg/day) [c]	11.5 ± 9.8 7.6 (15.4)	15.8 ± 29.3 4.4 (3.2)	0.70
Vitamin B6 (food) + vitamin B6 (dietary supplements) density (mg/1000 kcal) [d]	5.0 ± 4.1 3.4 (7.9)	9.0 ± 15.3 2.5 (3.6)	0.49

[a] Values expressed as mean ± standard deviation and median (interquartile range), except where noted. [b] Intake variable determined by 7-day weighed food records analyzed using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 20. [c] RDA for vitamin B6 for girls ages 14–18 = 1.2 mg/day. RDA for vitamin B6 for women ages 19–50 = 1.3 mg/day. [d] Recommended value for vitamin B6 density: 1 mg/1000 kcals [44]. * $p < 0.05$. ** $p < 0.01$.

Table 4. Vitamin B12 intakes (food and dietary supplements) in highly active and sedentary women [a].

Intake Variable [b]	**Activity Level**		***p*-Value**
	Highly Active ***n* = 29**	**Sedentary** ***n* = 29**	
Vitamin B12 intake from food			
Synthetic vitamin B12 (µg/day)	3.8 ± 5.8 2.7(1.6)	1.6 ± 2.2 0.7(1.8)	0.05
Vitamin B12 (µg/day) [c]	8.1 ± 6.3 6.1(5.4)	4.7 ± 2.4 4.3(2.0)	<0.01 **
Vitamin B12 density (µg/1000 kcal) [d]	3.6 ± 3.7 2.9(1.3)	2.7 ± 1.5 2.2(1.5)	0.21
Supplement contribution for those that reported supplement use			
Participants that reported vitamin B12 dietary supplement use (*n*(%))	9 (31)	12 (41)	
Vitamin B12 (dietary supplements) (µg/day)	34.8 ± 63.5 10.3(33.0)	36.4 ± 73.8 7.0(21.0)	0.96
Vitamin B12 (food) + vitamin B12 (dietary supplements) (µg/day) [c]	38.3 ± 66.0 10.4(35.6)	37.6 ± 74.0 9.1(21.6)	0.98
Vitamin B12 (food) + vitamin B12 (dietary supplements) density (µg/1000 kcal) [d]	15.9 ± 25.6 6.5(16.2)	23.7 ± 48.4 5.6(15.8)	0.66

[a] Values expressed as mean ± standard deviation and median (interquartile range), except where noted. [b] Intake variable determined by 7-day weighed food records analyzed using the United States Department of Agriculture (USDA) National Nutrient Database for Standard Reference, Release 20. [c] RDA for vitamin B12 for girls ages 14–18 = 2.4 µg/day. RDA for vitamin B12 for women ages 19–50 = 2.4 µg/day. Values include natural vitamin B12 and synthetic vitamin B12 added to food. [d] Recommended value for vitamin B12 density: 1.5 µg/1000 kcals [44]. * $p < 0.05$. ** $p < 0.01$.

Table 5. Evaluation of nutrient adequacy from food using DRI [a] recommendations of highly active and sedentary women.

Nutrient/DRI Factors	Activity Level		Reference Values: Girls 14–18 years [b]	Reference Values: Women 19–50 years [b]
	Highly Active	Sedentary		
	n = 29	*n* = 29		
Folate				
Met EAR (*n* (%)) [c]	28 (96)	26 (90)	330 µg/day	320 µg/day
Met RDA (*n* (%)) [d]	26 (90)	23 (79)	400 µg/day	400 µg/day
Exceeded UL (*n* (%)) [e,f]	4 (14)	1 (3)	800 µg/day	1000 µg/day
Vitamin B6				
Met EAR (*n* (%)) [c]	29 (100)	26 (90)	1.0 mg/day	1.1 mg/day
Met RDA (*n* (%)) [d]	27 (93)	25 (86)	1.2 mg/day	1.3 mg/day
Exceeded UL (*n* (%)) [e]	0 (0)	0 (0)	80 mg/day	100 mg/day
Vitamin B12				
Met EAR (*n* (%)) [c]	29 (100)	29 (100)	2.0 µg/day	2.0 µg/day
Met RDA (*n* (%)) [d]	29 (100)	27 (93)	2.4 µg/day	2.4 µg/day
Exceeded UL (*n* (%)) [e,g]	–	–	–	–

[a] DRI = Dietary Reference Intakes. [b] Reference value from Food and Nutrition Board, Institute of Medicine [18]. [c] EAR = Estimated Average Requirement. [d] RDA = Recommended Daily Allowance. [e] UL = Tolerable Upper Intake Level. [f] Because the UL for folate applies to synthetic forms (fortified foods, dietary supplements), this assessment only includes the contribution from fortified foods. [g] A UL for vitamin B12 has not been established.

Table 6. Evaluation of nutrient adequacy from food and supplements using the DRI [a] recommendations for the highly active and sedentary women that reported dietary supplement use.

Nutrient/DRI Factors	Activity Level		Reference Values: Girls 14–18 Yeras [b]	Reference Values: Women 19–50 Years [b]
	Highly Active	Sedentary		
Folate				
Participants that reported folate dietary supplement use	*n* = 9	*n* = 12		
Met EAR (*n* (%)) [c]	9 (100)	12 (100)	330 µg/day	320 µg/day
Met RDA (*n* (%)) [d]	9 (100)	12 (100)	400 µg/day	400 µg/day
Exceeded UL (*n* (%)) [e,f]	5 (56)	8 (67)	800 µg/day	1000 µg/day
Vitamin B6				
Participants that reported vitamin B6 dietary supplement use	*n* = 8	*n* = 12		
Met EAR (*n* (%)) [c]	8 (100)	12 (100)	1.0 mg/day	1.1 mg/day
Met RDA (*n* (%)) [d]	8 (100)	12 (100)	1.2 mg/day	1.3 mg/day
Exceeded UL (*n* (%)) [e]	0 (0)	1 (8)	80 mg/day	100 mg/day
Vitamin B12				
Participants that reported vitamin B12 dietary supplement use	*n* = 9	*n* = 12		
Met EAR (*n* (%)) [c]	9 (100)	12 (100)	2.0 µg/day	2.0 µg/day
Met RDA (*n* (%)) [d]	9 (100)	12 (100)	2.4 µg/day	2.4 µg/day
Exceeded UL (*n* (%)) [e,g]	–	–	–	–

[a] DRI—Dietary Reference Intakes. [b] Reference value from Food and Nutrition Board, Institute of Medicine [18]. [c] EAR—Estimated Average Requirement. [d] RDA—Recommended Daily Allowance. [e] UL—Tolerable Upper Intake Level. [f] Because the UL for folate applies to synthetic forms (fortified foods, dietary supplements), this assessment only includes the contribution from fortified foods and dietary supplements. [g] A UL for vitamin B12 has not been established.

Table 3 outlines the vitamin B6 intake from food and dietary supplements for the 58 highly active and sedentary women. Significant differences between groups were found in vitamin B6 intake from

food (mg/day) ($p < 0.01$) and vitamin B6 density from food (mg/1000 kcal) ($p = 0.03$). Eight highly active and 12 sedentary women reported the use of supplements containing vitamin B6. However, there were no significant differences between groups for vitamin B6 intakes for those participants that used dietary supplements.

Dietary and supplemental intake of vitamin B12 is summarized in Table 4. The highly active women reported a significantly higher intake of vitamin B12 from food (μg/day) ($p < 0.01$) than the sedentary women. No significant differences were found between groups for synthetic vitamin B12 added to food (μg/day) and vitamin B12 density (μg/1000 kcal) from food. Nine highly active women reported the use of supplements containing vitamin B12 compared to 12 sedentary participants. However, there were no significant differences between groups for vitamin B12 intakes for those participants that reported using dietary supplements.

Table 5 describes the adequacy of dietary micronutrient intake as set by the DRI recommendations for folate, vitamin B6, and vitamin B12. Ninety-six percent of the highly active women and 90% of the sedentary participants met the EAR for folate. Ninety percent of the highly active women and 79% of the sedentary women met the RDA for folate. Fourteen percent of the highly active women and 3% of the sedentary women exceeded the UL (Tolerable Upper Intake Level) for folate, a guideline that only applies to synthetic forms of the nutrient (i.e., fortified foods, dietary supplements). Thus, folic acid from fortified foods was the form of folate used in this estimation. For vitamin B6, 100% of the highly active women and 90% of the sedentary women met the EAR. Ninety-three percent of the highly active women and 86% of the sedentary women met the RDA for vitamin B6. The UL was not exceeded by either group of women for vitamin B6. For vitamin B12, 100% of the highly active women met the EAR and RDA. One hundred percent of the sedentary women met the EAR for vitamin B12 and 93% of the sedentary women met the RDA. Currently, a UL for vitamin B12 has not been established.

Table 6 describes the adequacy of dietary micronutrient intake from food and dietary supplements as set by the DRI recommendations for folate, vitamin B6, and vitamin B12 for those participants reporting dietary supplement use. All of the highly active and sedentary participants met the EAR and RDA for folate, vitamin B6, and vitamin B12 when including both food and dietary supplements. However, 5 of the highly active (56% of those reporting dietary supplement use) and 8 of the sedentary women (67% of those reporting dietary supplement use) exceeded the UL for folate, a guideline referring to synthetic folate sources (i.e., fortified foods, dietary supplements). None of the highly active woman exceeded the UL for vitamin B6, whereas 1 sedentary woman did.

3.3. Blood Biochemical Assessment

Table 7 summarizes biomarkers of B-vitamin status for the two groups of women. No differences were found between groups for any of the biomarkers for folate, vitamin B6, and vitamin B12. None of the participants had a plasma folate, red blood cell folate, or plasma vitamin B12 concentration below the reference range. However, two (7%) of the sedentary participants had plasma transcobalamin II concentrations below the reference range. Two (7%) of the highly active women and two (7%) of the sedentary participants had plasma transcobalamin II concentrations above the reference range. Additionally, two (7%) of the sedentary participants had PLP concentrations below the reference range.

Table 7 also describes the hematological data for the study participants. One athlete had a mean corpuscular volume (MCV), hemoglobin, and hematocrit values below the reference range. No participant was found to have an MCV value above the reference range. However, there were no significant differences between groups for these three hematological markers and CRP. Thirty-four percent of the athletes and 28% of the sedentary participants had CRP values above the reference value.

Table 7. Biochemical markers of highly active and sedentary women [a,b].

Blood Parameter	Reference Range	Activity Level		p-Value
		Highly Active	Sedentary	
		n = 29	n = 29	
Folate [c]	>3 ng/mL	11 ± 4	11 ± 4	0.91
Number below reference range (n (%))		0 (0)	0 (0)	
Red blood cell folate [c]	>140 ng/mL	444 ± 83	436 ± 122	0.79
Number below reference range (n (%))		0 (0)	0 (0)	
Vitamin B12 [c]	>170 pg/mL	647 ± 267	552 ± 168	0.11
Number below reference range (n (%))		0 (0)	0 (0)	
Transcobalamin II [d]	13–244 pg/mL	148 ± 115	146 ± 82	0.92
Number below reference range (n (%))		0 (0)	2 (7)	
Number above reference range (n (%))		2 (7)	2 (7)	
Pyridoxal 5′-phosphate [c]	>20 nmol/L	53 ± 34	45 ± 26	0.33
Number below reference range (n (%))		0 (0)	2 (7)	
Homocysteine [c]	<14 µmol/L	6 ± 2	6 ± 2	0.93
Number above reference range (n (%))		0 (0)	0 (0)	
Mean corpuscular volume [e]	78–100 fL	89 ± 6	89 ± 3	0.91
Participants below the reference range (n (%))		1 (3)	0 (0)	
Hemoglobin [e]	11.5–16.0 g/dL	13.5 ± 1.1	13.8 ± 0.7	0.23
Participants below the reference range (n (%))		1 (3)	0 (0)	
Hematocrit [e]	35%–48%	40 ± 3	40 ± 2	0.78
Participants below the reference range (n (%))		1 (3)	0 (0)	
High sensitivity C-reactive protein [e] median (interquartile range)	<1.0 mg/L	0.5 (3.2)	0.4 (1.0)	0.55
Participants above the reference range (n (%))		10 (34)	8 (28)	

[a] Mean ± standard deviation, except where noted. [b] Independent sample t-tests used to examine differences for all parameters except C-reactive protein (Mann Whitney U test). [c] Reference value from Food and Nutrition Board, Institute of Medicine [18]. [d] Reference value from Herzlich and Herbert [37]. [e] Reference value from Sonora Quest Laboratories. * $p < 0.05$.

4. Discussion

This study is one of few that summarizes a comprehensive nutrition assessment of the B-vitamins using dietary (food and dietary supplements) and biochemical assessments in highly active women compared to a control group of sedentary women. As part of the dietary assessment, food records were collected over 7 days (longer than other studies in the research literature) to determine nutrient adequacy using the DRIs for folate, vitamin B6, and vitamin B12. The average micronutrient intake of the highly active and sedentary participants not only met the 1998 DRIs, but were much higher than the dietary intakes reported in previous studies. Additionally, four highly active women and one sedentary participant exceeded the UL for folate with the consumption of fortified foods. Information was collected on dietary supplement use as part of the dietary assessment. The participants that used dietary supplements met the 1998 RDA for folate, vitamin B6, and vitamin B12. Furthermore, five highly active and eight sedentary women exceeded the UL for folate when the intake included both food and supplements. Dietary supplement use has not always been reported or included in previous studies. As part of the biochemical assessment, biomarkers for folate, vitamin B6, and vitamin B12 were determined. The mean values for the biomarkers were not significantly different between the two groups of women. All of the highly active women had biomarkers for folate, vitamin B6, and vitamin B12 within the reference ranges, suggesting good status. However, two sedentary women had low transcobalamin II concentrations, suggesting poor status of vitamin B12, and two different sedentary women had low PLP concentrations, suggesting poor status of vitamin B6.

4.1. Dietary Assessment

Folate, vitamin B6, and vitamin B12 intakes from food were significantly higher in the highly active women compared to the sedentary women. This finding may be related to the significantly higher energy intake by the highly active women. Only vitamin B6 was significantly different between groups for nutrient density, with a higher density in the highly active women; however, nutrient density

recommendations were met for all three nutrients by both groups. Thus, the highly active women were not necessarily consuming more nutrient dense foods, especially for folate and vitamin B12. Athletes with lower energy requirements may benefit from nutrition education to help them select more nutritious foods in the diet. The average dietary intake of vitamin B6, folate, and vitamin B12 for the women in our study exceeded the 1998 RDAs for folate (400 μg/day of DFE), vitamin B6 (1.2 mg/day for girls 14–18 years; 1.3 mg/day for women 19–50 years), and vitamin B12 (2.4 μg/day). The reported dietary intakes from this study are also much higher than the intakes reported in recent NHANES data (i.e., women 20–29 years of age: vitamin B6 = 1.91 mg, folate = 471 μg DFE, vitamin B12 = 4.23 μg) [16].

In our study, the dietary intakes for folate are higher than those reported by female athletes in previous studies conducted after the mandatory folic acid fortification [13,14,23,26]. For example, 25 synchronized figure skaters reported average intakes for folate of 65% of the 1998 RDA [14]. In another study, pre- and post-season intakes for folate were examined in 13 intercollegiate female soccer players [13]. Pre-season intakes were 271 $\pm$ 130 μg/day, while the post-season intake was reported as 186 $\pm$ 113 μg/day. Similarly, Leydon and Wall examined dietary intakes among female jockeys and reported average dietary intakes of only 132 $\pm$ 52 μg/day [23]. Among female recreational athletes, Joubert and Manore reported higher dietary intakes for folate of 428 $\pm$ 125 μg/day for female athletes participating primarily in low intensity activities and 511 $\pm$ 105 μg/day for female athletes participating in high intensity activities [26].

Similarly, the reported vitamin B6 intakes in our study are higher than those reported by female athletes in the research literature [13,14,23,26]. Among female soccer players, the mean pre-season vitamin B6 intake met the 1998 RDA, but the mean post-season intake did not [13]. The post-season overall energy intake was less than the pre-season intake and could account for a decreased vitamin B6 intake. Ziegler et al. examined the vitamin B6 intake of female synchronized figure skaters utilizing 3-day food records of 123 athletes and reported results by age [14]. The mean values for all participants and the girls aged 14–18 years did not meet the 1998 RDA for vitamin B6. The lower intakes reported in this study may be due to lower energy intakes as figure skating is seen as a weight conscious sport. For female jockeys, another weight conscious sport, the average vitamin B6 intakes were 0.90 $\pm$ 0.49 mg/day, less than the 1998 RDA and EAR [23]. However, other research has reported adequate vitamin B6 intakes. For instance, Joubert and Manore reported mean vitamin B6 intakes from 2.2 $\pm$ 1.6 to 2.4 $\pm$ 0.7 mg/day in recreational athletes, above the current RDA of 1.3 mg/day for women 19–50 years of age [26].

In the research literature, the mean intakes of vitamin B12 for female athletes are lower than those found in our study [13,14,23,26]. For example, the average pre- and post-season vitamin B12 intakes of female soccer players were 4.5 $\pm$ 1.9 μg/day and 2.1 $\pm$ 1.7 μg/day, respectively [13]. The pre-season intake was also higher in dietary protein, which possibly influenced the average vitamin B12 intake. The post-season average intake did not meet the 1998 RDA, but was adequate when compared to the EAR, a guideline that may not be appropriate for an athlete. In female synchronized figure skaters, the average intake met the 1998 RDA [14]. However, younger skaters (14–18 years) had average intakes of 2.2 μg/day for vitamin B12, less than the RDA. Leydon and Wall reported dietary intakes of vitamin B12 less than the RDA among female jockeys (2.15 $\pm$ 1.07 μg/day) [23]. Joubert and Manore reported dietary intakes for vitamin B12 that exceeded the RDA among female recreational athletes (5.3 $\pm$ 2.5 to 5.3 $\pm$ 4.8 μg/day) [26]. Our highly active women reported much higher vitamin B12 intakes (active = 8.1 $\pm$ 6.3 μg/day; sedentary = 4.7 $\pm$ 2.4 μg/day), which may be attributed to a more complete dietary assessment, utilizing a dietary software database that also included synthetic food sources of vitamin B12.

The current study reported higher intakes of vitamin B6, folate, and vitamin B12 than most other studies. Several factors may have contributed to this finding. First, when the dietary software program was missing micronutrient information for the B vitamins, we used the USDA nutrient database to reanalyze the food records. The USDA nutrient database was more complete in regards to B-vitamin content, including synthetic forms of folate and vitamin B12. Other studies may have

used software with similar missing data, thus, leading to an underestimation of dietary intakes. Second, our participants reported average intakes of 867 ± 391 µg/day (active) and 595 ± 250 µg/day (sedentary) DFE for folate. Food records showed large quantities of ready-to-eat breakfast cereals consumed by both groups of women. Some participants consumed >3 cups per sitting with multiple sittings per day. Four (14%) of the highly-active and one (3%) of the sedentary women had folate intakes that exceeded the UL of 1000 µg/day. Among US adults, Yang et al. reported that 2.7% of adults consumed more than the UL of folic acid, a percentage similar to the sedentary women in our study [45]. Thus, the consumption of ready-to-eat cereal was most likely associated with the higher dietary intakes of folic acid.

Two studies in female athletes also included dietary supplements when completing a dietary assessment of B-vitamins [25,34]. Singh et al. examined dietary intakes of ultra-marathon runners and found the average intake of vitamin B6 from food was 2.6 ± 0.3 mg/day for the usual diet and 2.3 ± 0.3 mg/day for the pre-race diet [34]. Vitamin B6 intake jumped to 7.3 ± 2.2 mg/day (usual diet) and 7.0 ± 2.4 mg/day (pre-race diet) when food and supplement intakes were combined. Average dietary folate intakes of the ultra-marathon runners were 391 µg/day, which only met the 1998 EAR of 320 µg/day of DFE [34]. When supplemental folic acid was included, average intakes of dietary folate increased (629 ±102 µg/day for usual intake and 513 ± 92 µg/day for pre-race intake). Singh et al. reported the average intake of vitamin B12 from food as 6.1 µg/day for the usual diet and 4.5 µg/day for the pre-race diet from food. Inclusion of supplemental vitamin B12 intake increased the total intake to 51.3 µg/day (usual diet) and 51.8 µg/day (pre-race diet), well above the 1998 RDA. Beshgetoor and Nichols also described the food and supplement intake of 25 female master cyclists and runners [25]. The mean intake of vitamin B6 for the supplementing athletes (SA) and the non-supplementing athletes (NSA) was 15 ± 5 mg/day and 3 ± 1 mg/day, respectively. Both groups met the 1998 RDA. The mean intake of folate for the SA and NSA was 486 ± 55 and 402 ± 115 µg/day DFE, respectively. The mean vitamin B12 intake for the SA and NSA was 18 ± 5 and 6 ± 2 µg/day, respectively, well above the EAR and RDA. Similarly, our study also documented higher consumption of folate, vitamin B6, and vitamin B12 for the highly active and sedentary women when both food and dietary supplements were included in the nutrient totals as part of the dietary assessment.

4.2. Biochemical Assessment

As part of the biochemical assessment, we examined biomarkers for folate, vitamin B6, and vitamin B12 in highly active and sedentary women. There were no significant differences between groups for any of the B-vitamin biomarkers. All of the highly active women had biomarkers within the reference ranges for plasma folate, RBC folate, plasma vitamin B6, vitamin B12, and homocysteine. Two sedentary women had transcobalamin II concentrations below the reference range of 13–244 pg/mL, suggesting poor vitamin B12 status from the biochemical assessment. When examining their dietary assessment data, neither participant reported using dietary supplements and one reported a vitamin B12 intake much lower than the group mean (2.8 µg/day). Two of the sedentary women had PLP concentrations <20 nmol/L, suggesting poor status of vitamin B6. Upon further examination of their dietary assessment data, one was non-supplementing and reported an average vitamin B6 intake of 1.35 mg/day. The other sedentary participant reported using a dietary supplement containing vitamin B6 (2 mg/day) and was consuming on average 1.54 mg/day of vitamin B6 from food. Ten of the highly active women had elevated CRP concentrations, which may be a sign of inflammation due to over training [46–48]. Eight of the sedentary women had elevated CRP levels as well, which may be related to environmental factors, such as stress at home or work, pollution, illness, or other factors. The more than adequate dietary intakes of folate, vitamin B6, and vitamin B12 certainly impacted the nutrient biomarkers.

Plasma homocysteine concentrations, a functional biomarker of B-vitamin status, were within the normal range in our highly active and sedentary women and there were no differences between the groups of women. The amount of training performed by our participants did not impact plasma

homocysteine concentrations. Similarly, the adequate dietary intakes for folate, vitamin B6, and vitamin B12 may have influenced plasma homocysteine concentrations. Some studies have documented acute increases in homocysteine after exercise, but then a return to baseline after a recovery or resting period. For instance, Wright et al. reported homocysteine concentrations in men increased immediately after a 30 minute bicycle ride, but began to decrease within 30 min after exercise [49]. In another study involving winter athletes, plasma homocysteine concentrations were higher during training and competition compared to baseline [50]. Dehydration and decreased blood volume after strenuous exercise may be a factor in these study results. Similarly, Gelecek and colleagues reported increases in homocysteine concentrations from baseline after one exercise session and a 6 week exercise training program [51]. These results can also be a factor of dehydration. Our study documented biomarkers for folate, vitamin B6, and vitamin B12 within the reference range for the highly active women, but we did not examine the effect of acute physical activity on these parameters.

4.3. Limitations

The first limitation is related to the self-reported food and activity logs. Participants were instructed how to complete the forms and provided written information to use as a guide. However, many food records needed clarification of contents, amounts consumed, and preparation. Participants may have omitted some of their dietary intake to make it look as though they consumed less. Examples include omitting “unhealthy” foods and reporting intake of more socially desirable foods. The second limitation is that the results of this study are not applicable to all female athletes as we did not have representatives from every sport discipline. Due to the small sample sizes included in our study (basketball, n = 1; cross country/long-distance running, n = 3; gymnastics, n = 1; ice hockey, n = 1; softball, n = 3; swimming, n = 11; tennis, n = 4; volleyball, n = 5), our results may not be generalizable to female athletes participating in these same sports. A third limitation is the classification of activity level using self-report of programmed physical activity. For instance, the sedentary women could have reported engaging in <2 h of programmed physical activity but have jobs that require them to stand, walk, and complete physical movement throughout the day (i.e., childcare, retail sales, landscaping), thus confounding the results. Fourth, due to limited research, power calculations were not computed for reported vitamin B12 dietary intakes and B-vitamin biomarkers. The study was not powered to detect differences between the active and sedentary women in reported dietary folate intakes and may not have been sufficiently powered to detect differences in the other study outcome measures. This low statistical power reduces the chance of detecting any true differences between the study groups and increases the likelihood of making a type II error. Thus, the study results should be interpreted with caution and may not be generalizable to other groups of highly active and sedentary women. Fifth, selection bias may have influenced the study results. Because the participants volunteered for this nutrition study, they may be more interested in health and nutrition than their peers and may not appropriately represent other highly active and sedentary young women. Another limitation is whether the participants followed the parameters of the fasting blood draw (8-h fasting). Participants were asked the time when food/meal was consumed before the blood draw. There is also the possibility the participants did not refrain from physical activity or smoking (if they reported social smoking) for 48 h prior to the blood draw. Acute bouts of strenuous exercise may have an impact on CRP levels and smoking may alter folate status and CRP concentrations [46–48,52].

4.4. Future Studies

This study is one of few to comprehensively assess B-vitamin status (folate, vitamin B6, and vitamin B12) using both dietary (food, dietary supplements) and biochemical assessments in highly active and sedentary women. We did not report losses of the B-vitamins or complete a clinical exam looking for physical signs and symptoms related to B-vitamin status. Additional biomarkers (both static and functional) could further describe B-vitamin status of highly active women. Future studies should also incorporate larger sample sizes representing athletes from varied sports.

5. Conclusions

This study described a comprehensive nutrition assessment of the B-vitamins in highly active and sedentary women. Although the highly active women had significantly higher dietary intakes of energy, folate, vitamin B6, and vitamin B12, there were no significant differences between groups for the biomarkers of B-vitamin status. The dietary intakes reported by the women in this study were much higher than the dietary intakes reported in the research literature. Additionally, 5 women (4 highly active, 1 sedentary) exceeded the UL for folate with the consumption of fortified foods. In this study, all of the women that used dietary supplements met the 1998 RDA for folate, vitamin B6, and vitamin B12. However, the UL for folate was exceeded by 5 highly active and 8 sedentary women when the intake included both food and supplements. Furthermore, all of the highly active women had biomarkers of B-vitamin status within the reference ranges, reflecting the adequate dietary intakes (food, dietary supplements) for folate, vitamin B6, and vitamin B12.

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4

Periodization of Carbohydrate Intake: Short-Term Effect on Performance

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Abstract: Background: "Sleep-low" consists of a sequential periodization of carbohydrate (CHO) availability—low glycogen recovery after "train high" glycogen-depleting interval training, followed by an overnight-fast and light intensity training ("train low") the following day. This strategy leads to an upregulation of several exercise-responsive signaling proteins, but the chronic effect on performance has received less attention. We investigated the effects of short-term exposure to this strategy on endurance performance. Methods: Following training familiarization, 11 trained cyclists were divided into two groups for a one-week intervention—one group implemented three cycles of periodized CHO intake to achieve the sleep-low strategy over six training sessions (SL, CHO intake: 6 $g \cdot kg^{-1} \cdot day^{-1}$), whereas the control group consumed an even distribution of CHO over the day (CON). Tests were a 2 h submaximal ride and a 20 km time trial. Results: SL improved their performance (mean: +3.2%; $p < 0.05$) compared to CON. The improvement was associated with a change in pacing strategy with higher power output during the second part of the test. No change in substrate utilization was observed after the training period for either group. Conclusion: Implementing the "sleep-low" strategy for one week improved performance by the same magnitude previously seen in a three-week intervention, without any significant changes in selected markers of metabolism.

Keywords: carbohydrate; performance; training; cycling time trial; trained athletes; lipid oxidation; perception of effort

1. Introduction

Carbohydrate-based fuels (CHO) are the main substrates used by the brain and skeletal muscle during exercise. Thus, nutritional recommendations for competition performance promote strategies to achieve "high CHO availability", in the form of adequate pre-exercise glycogen concentrations and additional CHO intake during competition to meet the specific fuel needs of the event [1,2]. However, recent research has provided new insight into the interactions of exercise with "low CHO availability", whereby the adaptive responses to training or recovery are enhanced in an environment of low exogenous and endogenous CHO stores [3]. Within this framework, glycogen is not only considered as an energetic substrate, but more as a regulator of metabolic signaling responses [4].

The aim of training is to act as a chronic stimulus leading to physiological adaptations and an improvement in performance. The acute and chronic effect of endurance exercise on metabolic responses have already been widely described and include mitochondrial biogenesis, shifts in fiber composition toward type I fibers, and enhanced oxidative metabolism [5,6]. Substrate availability interacts with the contractile stimulus to modulate these physiological responses to training [7] Specifically, muscle glycogen content can modulate physiological adaptations induced by endurance training by upregulating transcription factors and regulators of gene expression such as *PGC-1α* [8] and *p53* [9]. Based on these observations, a growing interest in training under conditions of low glycogen availability and/or low exogenous glucose availability has developed [3].

Several studies have reported that commencing a training session with low glycogen availability enhances expression of genes involved in mitochondrial biogenesis and substrate metabolism [10–13]. However, these studies have typically failed to show improvements in performance, likely because the beneficial "molecular" effects are negated by a decreased ability to sustain high intensity exercise under the conditions of low CHO availability [12,13]. This has led to interest in a "periodized" approach to CHO availability in the training program, where sessions undertaken to promote adaptation are carefully integrated with others focused on high quality performance outcomes. The "sleep-low" (SL) strategy represents one such sequence of periodized CHO availability, which allows athletes to perform high intensity training sessions supported by high CHO availability while enhancing metabolic adaptation associated with low glycogen availability [14–17].

Specifically, this strategy consists of a cycling of (1) late afternoon scheduling of a high intensity training (HIT) session undertaken with high glycogen stores; (2) withholding of the ingestion of CHO after the session to maintain glycogen depletion during the overnight recovery period; and (3) a low–moderate intensity steady-state exercise session (LIT) in the following morning completed after an overnight fast. Previous studies have reported that this strategy leads to increased activity of several proteins with putative roles in training adaptation (AMPK, p38 MAPK, p53) [9,14] and higher rates of fat oxidation during submaximal exercise [14]. However, the effects on endurance performance are equivocal. Recently, we [15] reported that integrating SL strategy, three times a week, during a three-week training intervention (i.e., nine occurrences of the sequence) was associated with an improved endurance performance in well-trained subjects (+3% during a 10 km running trial), coupled with an increase in submaximal cycling efficiency. A control group, who undertook the same training program with a similar total intake of energy and CHO, but normally distributed over the day, failed to improve performance. Furthermore, the performance improvements achieved by the SL program were associated with a decrease in body fat (−1.05%) [15] without any negative impact on immune function or sleep quality [16]. The original concept underlying this strategy is the periodization of the CHO intake: instead of a chronically low CHO intake, which has been shown to alter glycogen metabolism [18], high-intensity training sessions are performed under conditions of high glycogen availability. The recovery period, which plays a central role in the development of training adaptation [19], is non optimal for prolonging the period of optimized response to the training stimulus [20]. Lower-intensity training (LIT) is performed while fasted to maximize cellular adaptations and enhance rates of lipid oxidation.

Although the intervention in our three-week study was successful in improving performance and body composition [15], we note challenges to the feasibility of free-living athletes achieving the required dietary manipulations and/or having the commitment to undertake the low CHO recovery and subsequent training [21]. It is therefore of interest to see if a shorter exposure to this CHO periodization strategy would be successful in inducing metabolic adaptations and performance improvement. Accordingly, the aim of the current study was to investigate the effect of an abbreviated program of the "sleep-low" strategy on endurance performance in well-trained athletes. We also examined whether any observed effects on performance are related to an enhancement of metabolic adaptations to training as previously suggested [3].

2. Materials and Methods

2.1. Study Population

Eleven endurance-trained male cyclists volunteered to participate in the study. They were healthy, aged between 18 and 40 years, and training at least 12 h/week, having at least 3 years of prior training. Their mean (±SD) age was 31.2 ± 7.1 years, their mean body mass was 71.1 ± 5.6 kg, their mean maximal oxygen consumption ($\dot{V}O_{2max}$) was 64.2 ± 6.0 mL·min^{-1}·kg^{-1}, and their mean maximal aerobic power (MAP, W) was 342 ± 38.3 W. Before entering the study, all participants were examined by a cardiologist to ensure they did not present with abnormal electrocardiograph pattern or contraindications to physical activity. The study's protocol was approved by local Ethic Committee 2015-AO1136-43 (Paris IDF X, France). After written and verbal explanation, all participants provided their written informed consent to participate.

2.2. Study Design

An overview of the study design is depicted in the Figure 1. Subjects were first assigned to a familiarization session to the testing protocol. Then, during the following two weeks, they trained according to their habitual training program. During the first week, they ate according to their usual dietary habits, documenting their food intake via a daily food diary. In the second week, they followed specific nutritional guidelines, which set their CHO intake at 6 g·kg^{-1}·day^{-1}, while continuing to keep their daily food diary. After the two weeks of habitual training load, subjects were assigned to the PRE test session. Then, they were randomly assigned to two different groups undertaking the same one-week training program but following different nutritional guidelines, according to the "sleep-low" strategy, previously described [14,15]. CHO intake was similar between groups (6 g·kg^{-1}·day^{-1}) but periodized differently over the day, according to the demands of the training sessions. Specifically, one group trained with a high CHO availability (control group, CON group, n = 9) with an even spread of CHO intake over the day and between training sessions. Meanwhile, the intervention group trained with a CHO intake that was periodized within the various days ("sleep-low" group, SL group, n = 12) such that no CHO was consumed between the high intensity interval training sessions (HIT) held late in the day and the end of the following morning's low–moderate intensity (LIT) training session. The protocol ended with a POST test session.

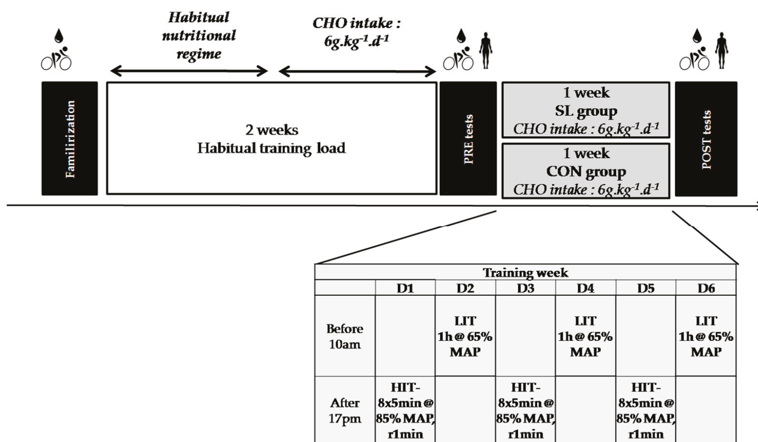

Figure 1. Overview of the experimental protocol; CHO: carbohydrates; HIT: high-intensity training session; LIT: light intensity training session; SL: Sleep-Low; CON: Control; MAP: Maximal aerobic power.

Since it was not possible to disguise the differences in dietary intake between the groups, this study could not be performed as a blinded intervention. In order to limit this bias, participants were not informed of the aim of the study (periodization of the CHO intake). They were neither aware of the number of groups in the study, the group to which they had been assigned, nor the program of the other group.

2.3. Preliminary Measurement of Maximal Oxygen Consumption

Before entering the study, all participants had to perform a $\dot{V}O_{2max}$ test, which was determined by an incremental test until exhaustion, on an electrically braked cycle ergometer (Excalibur Sport, Lode, Groningen, The Netherlands). Saddle and handlebar heights were set to match the usual positions used by participants, and these were standardized between sessions. The cycle ergometer was equipped with individual racing pedals, allowing participants to wear their own shoes. Subjects warmed up for 6 min at 100 W, then power output was increased by 25 W each successive 2 min until volitional exhaustion. Participants wore a face mask covering their mouth and nose to collect breath (Hans Rudolph, Kansas City, MO, USA). During the test, oxygen uptake ($\dot{V}O_2$), carbon dioxide uptake ($\dot{V}CO_2$), minute ventilation ($\dot{V}E$) and the respiratory exchange ratio (RER) were continuously recorded and monitored as breath-by-breath values (Quark, Cosmed, Rome, Italy). The gas and flow analyzers were calibrated prior to each test using ambient air, known-concentration gas (O_2 = 16%, CO_2 = 5%), and a 3 L syringe. The $\dot{V}O_{2max}$ was determined based on the highest 30 s average value. The MAP (W) was calculated as MAP = W completed + 25 × (t/120), where W is the last completed workload and t is the number of seconds in the last workload [22]. The MAP was used to adjust the workload in the testing session and the training program.

2.4. Training Protocol

The training program was divided in two phases. The first phase, lasting two weeks, was composed of the participants' habitual training programs. The second phase lasted one week and was similar for all participants, regardless of the nutritional group to which they were assigned. The training program (Figure 1) was based on our previous studies [15,16] and consisted of six training sessions over six consecutive days, including a HIT session in the afternoon (after 1700 h) and low–moderate intensity training session in the following morning (before 1000 h). The HIT session comprised a 10 min warm-up followed by eight repetitions lasting 5 min at 85% of MAP interspersed with 1 min of recovery (100 W). The cycling LIT sessions consisted of a steady-state 1 h session at 65% of MAP.

2.5. Nutritional Protocol

During the first week of the protocol of the habitual training load, participants were not assigned to specific nutritional guidelines. They were asked to complete a food diary in order to record their nutritional habits and examine how they differed from the nutritional interventions applied in the study. The second week of this first phase of the protocol, all participants were given dietary prescriptions, setting CHO intake at 6 $g \cdot kg^{-1} \cdot day^{-1}$ in anticipation of the nutritional strategy of the second phase. Participants were given precise instructions for the weighed food allowances for each meal (breakfast, lunch, dinner, and during training) according to their body mass. During the week of modified training program, participants were separated into two groups: the CON group (n = 9) and the SL group (n = 12). They were instructed to ingest the same amount of CHO during the day (6 $g \cdot kg^{-1}$) but spread differently over the day (Table 1). A full description of the dietary program can be found elsewhere [16]. Briefly, for the SL group, no CHO was consumed from the commencement of the HIT session on the evening of one day until after the completion of the LIT session on the following morning. Thereafter, CHO intake was resumed to meet daily targets. In this way, the HIT session was undertaken with high muscle glycogen concentrations ("train-high"), while recovery from

this session and the completion of the LIT session was undertaken with low CHO availability due to depleted glycogen concentrations and an overnight fast ("sleep-low" and "train-low", respectively). Meanwhile, high glycogen availability was maintained in the CON group with regular intake of CHO at all meals throughout the day, and the intake of a sports drink (6% CHO, Gatorade, PepsiCo, Purchase, NY, USA) during training sessions. CHO was ingested at every meal. Each participant received written nutritional recommendations for each meal with quantities according to their group and weight. To prevent an unwanted loss of fat-free mass, a high-protein sugar-free drink (High Protein 15 g, UHS, Bruno, France) was prescribed before going to bed. To check compliance to the dietary protocols, participants were required to complete a daily food diary. They were instructed to give as many details as possible (food weights, pictures of dishes, descriptions of fat used to cook or flavor dishes, and the brand names of commercial food products). The diaries were inspected by the same researcher and analyzed using a self-made database of food composition.

Table 1. Total energy and macronutrient intake for sleep-low (SL) and control (CON) groups before starting the training program (BASELINE) and during the training/diet intervention (TRAINING) (mean ± SD).

		Total Energy Intake ($kcal \cdot Day^{-1}$)	Carbohydrate Intake ($g \cdot kg^{-1} \cdot Day^{-1}$)	Lipid Intake ($g \cdot kg^{-1} \cdot Day^{-1}$)	Protein Intake ($g \cdot kg^{-1} \cdot Day^{-1}$)
SL group	BASELINE	2658 ± 726	4.9 ± 1.3	1.2 ± 0.4	1.4 ± 0.4
n = 12	TRAINING	3079 ± 874	6.5 ± 2.2	0.9 ± 0.3	1.9 ± 0.2 *
CON group	BASELINE	2924 ± 967	5.2 ± 1.9	1.4 ± 0.5	1.4 ± 0.5
n = 9	TRAINING	2610 ± 488	5.0 ± 1.3	0.9 ± 0.3 *	1.6 ± 0.4

*: $p < 0.05$ as compared to PRE values.

Meals during the 24 h prior to the testing sessions (lunch, dinner, and breakfast) were identically prescribed for both groups to ensure that the same amount of CHO (total intake of 6 $g \cdot kg^{-1} \cdot day^{-1}$) was consumed.

2.6. Testing Protocol

Three sessions of testing were planned: familiarization, PRE, and POST tests. They were composed of two exercise sessions on the same day. The day after the last training session of the week, subjects reported to the laboratory at a standardized time. The first test was a 2 h submaximal cycling test at 60% of MAP at a self-selected cadence. The test started with 10 min at 100 W followed by 110 min at 60% of MAP. Participants wore a cardio belt to monitor heart rate (HR) constantly throughout the test, as well as a face mask to measure gas exchange. They wore the mask for the first 20 min and then the mask was removed for 10 min every 10 min, allowing the subjects to drink only water. Respiratory gases were collected and analyzed to assess cycling efficiency, substrate oxidation, and respiratory quotient. Specifically, whole body rates of CHO and fat oxidation (in $g \cdot min^{-1}$) were calculated from $\dot{V}O_2$ and $\dot{V}CO_2$ values measured during the submaximal cycling test; calculations were made from gases collected during the last 60 s of each work interval of interest with nonprotein respiratory exchange ratio (RER) values being assessed according to standard equations [23]:

$$\text{CHO oxidation} = 4.210\dot{V}CO_2 - 2.962\dot{V}O_2 \quad (1)$$

$$\text{Fat oxidation} = 1.695\dot{V}O_2 - 1.701\dot{V}CO_2 \quad (2)$$

Three blood samples were collected during the submaximal test—immediately before, at 1 h, and at 2 h of the test—from a superficial forearm using venipuncture techniques. Four 33 mL samples of blood were collected into EDTA and Z Serum Clot Activator tubes (Greiner Bio-One, Frickenhausen, Germany).

The submaximal test was immediately followed by a 20 km time-trial (TT) performed on the participants' own bike mounted on a braked Cyclus2 ergometer (RBM GmbH, Leipzig, Germany). We tried to reproduce realistic conditions of a cycling race, within a laboratory environment. Ingestion of sports drink (6% CHO, Gatorade, PepsiCo, Purchase, NY, USA) was allowed during the time-trial, with the volume ingested during the familiarization being recorded and replicated during the ensuing testing sessions. No feedback was provided to the subjects during TT except for their gear ratio and the distance remaining. Rating perception of effort (RPE) was assessed verbally using the Borg 6–20 scale [24] every 5 km. Heart rate (HR) was continuously sampled every 5 s (Polar, Kempele, Finland) during the TT. The time, the mean power, and the mean speed were collected at the end of the TT. Pacing strategy was reported per kilometer during the TT. Participants were not informed of their results until the end of the study.

2.7. Blood Analysis

To avoid interassay variation, all blood samples were analyzed in a single batch at the end of the study. Blood samples were collected to measure plasma concentrations of markers of lipid metabolism (glycerol and free fatty acid) and markers of metabolic stress (adrenaline and noradrenaline). After collection, blood samples were immediately centrifuged at 4000 rev·min^{-1} for 10 min at 4 °C to separate plasma from red blood cells. Plasma was then stored in multiple aliquots (Eppendorf type, 1500 μL) at −80 °C until analysis. Catecholamine concentrations were determined with commercially available ELISA kits (Demeditec Diagnostics GmbH, Kiel, Germany). The assay for (adrenaline) had an intra-assay coefficient of variation (CV) of 24.7%–11.0% over a concentration range of 64.7–948 pg·mL^{-1} and an interassay CV of 14.5%–13.1% over a concentration range of 76.4–771 pg·mL^{-1}. The assay for noradrenaline had intra-assay CV of 12.8%–11.1% over a concentration range of 510–3363 pg·mL^{-1} and an interassay CV of 9.2%–9.2% over a concentration range of 445–3283 pg·mL^{-1}. All blood samples were analyzed in duplicate in respective wavelengths on a spectrophotometer Dynex MRXe (Legalla Biosciences, Chelmsford, MA, USA).

Plasma non-esterified fatty acids (NEFA) were determined with an enzymatic method (Wako Chemical, Neuss, Germany) and glycerol concentrations were measured with enzymatic colorimetric method Randox (Crumnil, Antrin, UK) on PENTRA 400 Horiba (ABX, Montpellier, France).

2.8. Body Composition

Measurement of whole body composition was undertaken on all subjects using dual-energy X-ray absorptiometry (Lunar IDXA, General Electric, Madison, WI, USA) at PRE and POST test sessions, the day after the performance tests. All measurements were taken early in the morning and in a fasted state [25].

2.9. Statistical Analysis

All statistical analyses were conducted using Statistica 7.1 software (StatSoft). All data are expressed as mean ± SD. Normality of data was tested using a Shapiro–Wilk normality test. Data which were not normally distributed were log-transformed. A repeated-measures analysis of variance (ANOVA) was used to calculate the effect of the dietary strategy (SL vs. CON) and the period (PRE and POST) on performance, blood parameters, and body composition. When a significant effect was found, post hoc tests were performed using Newman–Keuls procedures. Effect sizes for comparison were then calculated Cohen's d values. Values of 0.1, 0.3, and over 0.5 were respectively considered as small, medium, and large effect [26]. For all tests, the significance level was set at $p < 0.05$.

3. Results

3.1. Dietary Intervention

Analyses of food diaries revealed that participants complied with the nutritional guidelines of their prescribed diet (Table 1). There was no significant difference in the CHO intake between both groups before and after the training/diet intervention week, despite a slightly difference in the effective CHO intake. Total protein intake increased between the baseline training period and the training diet week (+36.3% and +20.4%, $p < 0.05$, d = 3.48 and d = 1.07, for SL and CON groups, respectively) but without any difference between groups. In both groups, there was also a reduction in reported intake of fat during the training/diet intervention period compared with baseline (−17.8% and −20.9%, $p < 0.01$, d = 2.13 and d = 2.81 for SL and CON groups, respectively).

3.2. Performance Tests

3.2.1. Twenty Kilometer Time-Trial Cycling Test Performance

Time to complete the 20 km cycling time-trial was reduced after the training period for all the subjects in SL (−3.23% ± 2.99%, $p < 0.05$, d = 1.58), whereas no change was recorded for CON (−1.04% ± 3.46%) (Figure 2). This improvement was due to a significantly higher mean power output (from 229 ± 36 to 250 ± 32 W, $p < 0.05$, d = 1.48) in SL.

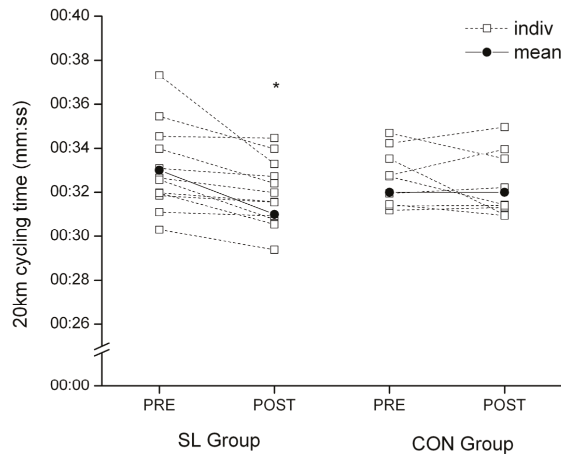

Figure 2. Individual 20 km cycling time-trial performance for SL and CON groups in PRE and POST tests. * Significantly different from PRE values, $p < 0.05$.

• Pacing strategy

The change in mean power over the duration of the time-trial is depicted in Figure 3. The SL strategy induced a significantly higher mean power at the 11th (+13.2% ± 15%, $p < 0.05$, d = 1.58), 13th (+18.1% ± 23.4%, $p < 0.01$, d = 1.95), 14th (+14.3% ± 14.6%, $p < 0.05$, d = 1.58), 15th (21.2% ± 12.8%, $p < 0.01$, d = 2.95), 16th (+11.8% ± 8.4%, $p < 0.05$, d = 1.92), and 17th kilometers (+12.4% ± 9.4%, $p < 0.05$, d = 1.74) (Figure 3a), whereas no change was observed after the training week for the CON group (Figure 3b). Both groups developed higher mean power at the 20th kilometer after the training week (+7.7% ± 14%, $p < 0.05$, d = 0.85 for SL group; +11.2% ± 20%, $p < 0.01$, d = 2.31 for CON group).

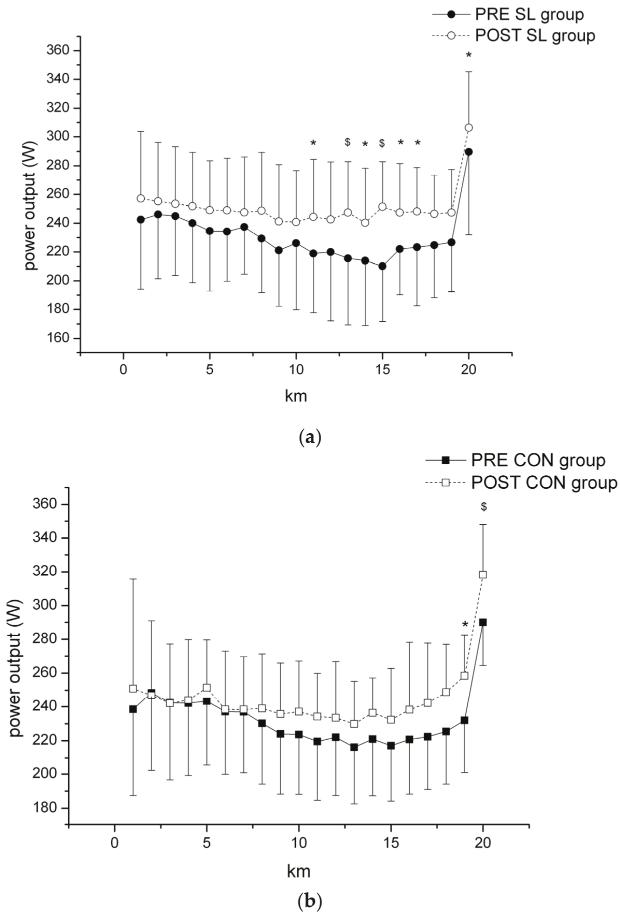

Figure 3. Pacing strategy (absolute change in power output per kilometer) during the 20 km cycling time-trial in PRE and POST tests for (**a**) SL group; and (**b**) CON group. * Significantly different from PRE values, $p < 0.05$. $ Significantly different from PRE values, $p < 0.01$.

- RPE

No difference in RPE values during the time trial was observed between PRE and POST tests for both groups (Table 2), despite the higher outputs of the SL group in the POST test trial.

Table 2. Rating perception of effort (RPE) during the 20 km cycling time-trial every 5 km for SL and CON groups in PRE and POST tests.

		RPE				
		0	**5 km**	**10 km**	**15 km**	**20 km**
SL group	PRE	9 ± 1.2	14.7 ± 2.3	16.2 ± 1.6	17.3 ± 1.7	19 ± 1.2
	POST	10 ± 2.5	15 ± 2	16 ± 1.6	17.2 ± 1.3	19 ± 1
CON group	PRE	10.9 ± 2	14.7 ± 1.7	15.3 ± 2.3	16.2 ± 2	17.7 ± 1.9
	POST	13.1 ± 2.8	14.3 ± 1.7	15 ± 2.4	16 ± 1.7	18 ± 1.7

3.2.2. Submaximal Cycling Test

• Substrate oxidation

No significant differences between group and pre and post tests was observed for rates of CHO oxidation (mean values during the whole test: respectively for pre and post test for the SL group 2.0 ± 0.2 g·min^{-1} vs. 2.1 ± 0.2 g·min^{-1}; and for the CON group: 1.9 ±0.5 g·min^{-1} vs. 2.1 ± 0.5 g·min^{-1}) or fat oxidation (respectively for pre and post test for the SL group 0.6 ± 0.3 g·min^{-1} vs. 0.9 ± 0.2 g·min^{-1}; and for the CON group: 0.7 ± 0.2 g·min^{-1} vs. 0.6 ± 0.2 g·min^{-1}).

• Blood analysis

Markers of lipid metabolism. Plasma concentrations of glycerol increased during the submaximal cycling test ($p < 0.001$) but differences between groups or between PRE and POST tests were not significant (Table 3). Similarly, there was an increase in plasma concentrations of free fatty acids during the test ($p < 0.001$) but without any difference between groups or between PRE and POST tests.

Markers of stress. Plasma catecholamine concentrations increased during the submaximal cycling test: the concentrations at 1 h and at 2 h were higher than resting concentrations for both groups ($p < 0.01$ for both markers). No significant difference in plasma catecholamine concentrations were observed before and after the training/diet intervention or between groups (Table 3).

Table 3. Blood analysis sampled before, during (at 1 h) and immediately after (at 2 h) the submaximal test for markers of lipid metabolism (glycerol, non-esterified fatty acid (NEFA)) and catecholamine concentrations.

		Glycerol (mmol·L^{-1})			NEFA (µmol·L^{-1})		
	Blood Sampling	**Before**	**During**	**After**	**Before**	**During**	**After**
SL group	PRE	0.02 ± 0.01	0.11 ± 0.06	0.25 ± 0.13	185 ± 115	308 ± 135	610 ± 209
	POST	0.02 ± 0.01	0.07 ± 0.04	0.22 ± 0.1	168 ± 79	229 ± 90	589 ± 213
CON group	PRE	0.03 ± 0.01	0.08 ± 0.03	0.21 ± 0.08	153 ± 60	241 ± 148	604 ± 284
	POST	0.03 ± 0.03	0.10 ± 0.05	0.22 ± 0.11	134 ± 59	341 ± 222	699 ± 457
		Adrenaline (ng·mL^{-1})			**Noradrenaline (ng·mL^{-1})**		
	Blood Sampling	**Before**	**During**	**After**	**Before**	**During**	**After**
SL group	PRE	0.10 ± 0.13	0.31 ± 0.25	1.1 ± 0.79	0.93 ± 0.92	4.17 ± 2.1 $	4.6 ± 3.8 $
	POST	0.07 ± 0.10	0.16 ± 0.17	0.73 ± 0.67 *	0.9 ± 0.6	3.8 ± 3.6 *	2.9 ± 2.2 *
CON group	PRE	0.18 ± 0.25	0.36 ± 0.1 $	0.27 ± 1.64 $	1.68 ± 1.0	4.13 ± 4.5	7.6 ± 4.4
	POST	0.04 ± 0.04	0.30 ± 0.13	0.48 ± 0.20	5.1 ± 7.2	10.1 ± 7.9	7.3 ± 6.9

$ significantly different from PRE before values, $p < 0.01$; * significantly different from POST before values, $p < 0.05$.

3.3. Training Period

The perception of effort for the LIT training session during the intervention was significantly different between groups. Subjects who trained in a fasted state (SL group) perceived the LIT training sessions as harder (15.2 ± 1.9) than the subjects of the CON group (13.5 ± 2) ($p < 0.05$, d = 0.87).

3.4. Body Composition

There were no differences in body mass and fat-free mass for either group after the intervention week. However, there was a significant reduction in fat mass in the SL group only (−395 ± 491 g, $p < 0.05$, d = 0.34), whereas the change observed in the CON group was not significant (−151 ± 363 g).

4. Discussion

This study investigated the effect of a short-term exposure to a periodized "sleep-low" training/diet strategy on metabolism and performance of well-trained cyclists. The program involved exposure to three cycles of a sequence involving "train high, sleep low, and train low" based on periodizing CHO intake to achieve different levels of CHO availability for specific training sessions within a week of training. The main finding was a significant improvement in performance during a cycling time-trial after only one week of training under the "sleep-low" strategy (+3.2% ± 2.99%). This improvement is similar in magnitude to that observed previously after three weeks of SL training [15]. No significant effect was observed for any other physiological parameter. This enhanced performance was related to differences in pacing strategy, and higher levels of self-chosen power outputs in the athletes who undertook the periodized CHO intake protocol. These findings show the importance of pacing in the determination of performance, and suggest factors other than physiological or metabolic characteristics that have been previously reported in studies focusing on the effect of low glycogen availability during training [7].

Strategies that promote training adaptation with low CHO availability (overnight-fasted training, low-glycogen training, low glycogen recovery periods) are commonly observed among athletes, but are often implemented unintentionally or without strategy. The lack of efficacy of these protocols in some studies [12,13] suggests that unless they are implemented in a strategic way, the outcomes may not integrate with other aspects of the training program towards a clear performance improvement. A case study describing the real-life training program of three elite marathoners during a 16-week training program [21] illustrated a sophisticated approach to mixing and matching specific training sessions with varying CHO availability, with the frequency of low CHO training varying from 1.3 to 2.6 sessions/week of training at different times of the season. Our protocol involves a specific sequence of three different training/nutrient stimuli, and this study brings new information regarding how they might achieve benefits in a shorter period or be scheduled at a strategic time before competition [27], at least in athletes of this well-trained but sub-elite caliber.

The improvement in performance in the current study was associated with change in the pacing strategy. Among the participants in our group who undertook the "sleep-low" exposure, self-chosen power outputs in the second half of the time-trial (11th–17th kilometer) were higher despite the same perceived exertion. Factors affecting pacing strategies have been widely investigated during the last decade and several models have been proposed [28–31].

It has been suggested that endurance performance is centrally regulated by both intrinsic (cognitive, mental fatigue, physiological) and extrinsic (environmental) signals to preserve physiological limits [32]. In the psychobiological model of Marcora [31], pacing regulation could be explained using an effort-based decision-making model based on motivational intensity theory. This model states that the conscious regulation of pace is determined by five cognitive factors: (1) perception of effort; (2) potential motivation; (3) knowledge of the distance/time to cover; (4) knowledge of the distance/time remaining; and (5) previous experience of perception of effort during exercise of varying intensity and duration. In most of the cases, perception of effort is the key determinant of these models. In any event, the pacing strategy is adopted very rapidly, meaning that it is not only a function of metabolic changes [30].

One hypothesis to explain the impact of the periodization of CHO intake on the improvement of performance could reside in changes in resting muscle glycogen concentration. In a twice-daily training model in which the second session was undertaken with low glycogen availability, Hansen et al. and Yeo et al. [11,12] found a higher resting glycogen content in muscle that had received this exposure. It is possible that the participants in the SL group achieved an enhancement of glycogen storage leading to higher muscle glycogen concentration at the start of the 20 km time-trial. Muscle glycogen depletion, when the athlete is fed, is correlated to the development of fatigue [33]. The lower values of RPE after the training period can also be explained by higher muscle glycogen concentration. Rauch et al. [34] proposed that the power output developed is dependent on the brain, which anticipates the rate

of muscle glycogen utilization leading to individual "critical" levels of endpoint muscle glycogen. In their study, eight subjects followed three days of carbohydrate loading or a normal diet with an exercise protocol in which they completed 2 h cycling at 65% of MAP interspersed with five 60 s sprints after 20, 40, 60, 80, and 100 min. This bout was followed immediately by a time-trial of 1 h. Although the power outputs developed in the trial following the normal diet were lower than those in the carbohydrate loading trial, endpoint muscle glycogen concentrations were similar in both conditions, despite different starting concentrations. Although we were unable to measure muscle glycogen in our study, it is possible that higher pre-exercise muscle glycogen concentrations in the SL group may "signal" to the brain to allow higher power output. Future studies should investigate this hypothesis.

One limitation of our study which could also explain the possibly higher muscular glycogen content is the trend for an increase in energy and CHO intake for the SL group between PRE and POST testing sessions, while it was slightly reduced for the CON group. We note that although we provided precise nutritional guidelines to participants, they were free-living and prepared their own meals. Therefore, slight deviations from the desired dietary control could have possibly induced a bias in the outcomes. It should be noted, however, that despite these trends in reported energy intake, the SL group reported a small decrease in fat mass over the intervention period.

Another interesting finding of our study is that the performance improvement seen in the SL group was not associated with the metabolic changes classically reported after training with low CHO availability [14]. No changes in fat oxidation were observed during the submaximal cycling bout in the SL participants, while blood analyses also failed to record any change in metabolites or catecholamine levels after one week of "sleep-low" training strategy. The lack of any effect of the SL strategy on substrate oxidation is similar to the findings of our first study using a three-week SL strategy [15], but contrasts with the observations from previous studies on training with low glycogen availability. Typically, these studies report higher activity of enzymes involved in fat metabolism [12,13], and changes in transcription for adaptive genes [14] or factors involved in mitochondrial biogenesis [17]. However, a difference between our study and others is that our performance tests were undertaken pre- and post- intervention with subjects following strategies of high CHO availability (i.e., high CHO diet in the preceding day, pre-exercise CHO intake, CHO intake during the exercise). Thus, previous studies reported the effect of exercise in fasted conditions [10,35] as well as the effect of training with low CHO availability. In terms of effects on catecholamine concentrations, the lack of changes in the current study are consistent with the findings of our longer study, in which an increase in resting catecholamine concentrations was observed in the second and the third week of the training/diet intervention. This indicates that a longer period of exposure is needed to achieve measurable modifications in plasma catecholamine concentration.

5. Conclusions

One week of training with sequential periodization of CHO availability for selected periods of training (recovery, light intensity training session) seems sufficient to improve performance in trained endurance athletes. This strategy could be implemented during the weeks preceding a competition before the taper period.

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contributions to conception, design, acquisition of data, analysis and interpretation of data and has been involved in drafting the manuscript.

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5

"Eat as If You Could Save the Planet and Win!" Sustainability Integration into Nutrition for Exercise and Sport

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Abstract: Today's industrial food production contributes significantly to environmental degradation. Meat production accounts for the largest impact, including greenhouse gas emissions, land and water use. While food production and consumption are important aspects when addressing climate change, this article focuses predominantly on dietary change that promotes both health for planet and people with focus on athletes. Healthy, sustainable eating recommendations begin to appear in various governmental guidelines. However, there remains resistance to the suggested reductions in meat consumption. While food citizens are likely to choose what is good for them and the planet, others may not, unless healthy eating initiatives integrate creative food literacy approaches with experiential learning as a potential vehicle for change. This concept paper is organized in three sections: (1) Environmental impact of food; (2) health and sustainability connections; and (3) application in sports and exercise. For active individuals, this article focuses on the quantity of protein, highlighting meat and dairy, and quality of food, with topics such as organic production and biodiversity. Finally, the timing of when to integrate sustainability principles in sport nutrition is discussed, followed by practical applications for education and inclusion in team, institutional, and event operations.

Keywords: sustainability; food; environment; sports nutrition; athlete; health; sustainable diet; food literacy

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1. Introduction

There is an urgent need to reduce the degradation of natural resources and limit global warming, while providing healthy and sustainably produced food to a growing population. Agriculture contributes greatly to climate change and resource extraction, with animal-based foods playing a major role in greenhouse gas (GhG) emissions, loss of land, water, and biodiversity [1–4] Further, current dietary patterns contribute to chronic disease through inadequate intakes of plant-based foods and high consumption of red and processed meat [5,6]. In addition, climate change itself will negatively affect food production should temperatures continue to rise, resulting in reduced yields [7,8]—possibly as much as 30–40% loss by the turn of the century [9]. Adding to this, the consequential sea level rise due to ice melt in the Arctic, displacing not only people but also valuable agricultural land [10], and thus, indicating that food security will likely become the major threat to humans on earth. While agriculture itself must assume more sustainable practices, despite the continued need for intensification [11], strategies for adopting diets with lower environmental impact that are healthy, economically viable, and socially and culturally acceptable are also needed. Thus, for the first time in the history of dietary guidance, food and climate change are crossing paths,

and promoting a sustainable, healthful diet, also fit for the athlete, is now more than ever arising as an urgent public and planetary message.

This concept paper is organized in three sections: (1) Environmental impact of food; (2) health and sustainability connections; and (3) application in sports and exercise. For active individuals, this article focuses on the quantity of protein, highlighting meat and dairy, and the quality of food, with topics such as organic production and biodiversity. Finally, the timing of when to integrate sustainability principles in sport nutrition is discussed, followed by practical applications for education and inclusion in team, institutional, and event operations.

1.1. Environmental Impact of Food

The environmental impact of food production affects both terrestrial and marine environments. Agriculture uses about one third of arable land, almost three fourths of global water resources, and one fifth of energy. Thus, agriculture is a major contributor to resource depletion [12]. Agriculture also emits large quantities of GhGs. Agriculture accounts for 30% of total GhG emissions from pre-production, production, to post-production [7,13,14], with direct emissions from agriculture contributing the most [7].

Greenhouse gas emissions are quantified in terms of carbon dioxide equivalents (CO_2eq), collectively also known as global warming potential. Carbon dioxide (CO_2) is the most prominent anthropogenic GhG with a global warming potential of 1. Nitrous oxide (N_2O) and methane (CH_4) are the other two major GhGs, with global warming potential of over 300 and 25 times that of CO_2, respectively, expressed over a 100-year lifespan [15]. Thus, these GhGs contribute significantly to global warming, and therefore, are at least as important to mitigate as CO_2.

Direct emissions from agriculture account for the largest fraction in agriculture-related GhG emissions, by generating CO_2, N_2O, and CH_4 directly on the farm [7]. Nitrous oxide arises from fertilizer applied to soil, as part of the denitrification process. Agriculture produces 65% of all N_2O [16,17]. Methane is generated in large quantities from enteric fermentation and manure from ruminants [1,17–19] and, to a smaller extent, from rice production [14]. Further direct, on-farm emissions originate from fossil fuel dependence to run tractors and machinery, which release CO_2 [7,20]. Adding to this the high demand for animal feed, such as corn and soy, from agriculture, animal agriculture (especially ruminant) plays the biggest role in food-generated GhG emissions and global warming potential [19], exceeding the production of vegetables, grains, and legumes [21,22]. While direct emissions from agriculture contribute the greatest in global warming potential, pre-production processes also include resource-intensive fertilizer, pesticide and herbicide production, which emit GhGs [14]. Climate change mitigation, especially from direct emissions, is critical, as estimates indicate an additional 35–60% rise in CH_4 and N_2O already by 2030 [23]. Table 1 shows GhG emissions per kilogram of various foods.

Food production requires arable land, but there are not unlimited resources. About 33% of Earth's ice-free surface is used for agriculture [12]. Animal agriculture requires large amounts of land—approximately half of all of agriculture—not only for the animals, but also to produce their feed [1,19,24]. Agriculture has negatively affected the land, with excessive chemical input, causing poor soil health and pollution, with potential adverse human health effects [25–32]. While meat production has become industrial and inexpensive, its impact on animals and people have been largely neglected [19,33,34]. To meet a rising demand for food, especially meat, ecosystems continue to be compromised to clear more land [1,7]. This land clearing is also called deforestation and is an indirect but large contributor to agriculture's impact on the environment, including the loss of biodiversity [1,2,4].

Table 1. Greenhouse gas (GhGs) emissions in food.

Low GhGs	Medium GhGs	High GhGs
<1 kg CO_2 eq/kg edible weight	1–4 kg CO_2 eq/kg edible weight	>4 kg CO_2 eq/kg edible weight
Potatoes	Chicken	* Beef
Pasta	Milk, butter, yogurt	* Lamb
Bread	Eggs	Pork
Oats and other grain	Rice	Turkey
Vegetables (e.g., onions, peas, carrots, corn, brassica)	Breakfast cereals	Fish
Fruits (e.g., apples, pears, citrus, plums, grapes)	Spreads	Cheese
Beans/lentils	Nuts/Seeds	
Confectionary	Biscuits, cakes, dessert	
Savory Snacks	Fruit (e.g., berries, banana, melons, salad)	
	Vegetables (e.g., salad, mushrooms, green beans, cauliflower, broccoli, squash)	

* May be as high as 20–50 kg CO_2 eq/kg edible weight. Average CO_2 emissions for driving car are 0.186 kg CO_2 eq/km driven. Adapted from [35] (with permission).

Post-production GhG emissions include emissions from food storage, packaging, distribution, transport, and end-consumer effects (e.g., waste). Compared to agriculture's direct emissions, post-production GhG emissions are considered small [14,36]. Taken together, direct (on farm) and indirect (deforestation) effects of agriculture contribute the largest part of all food-related GhG emissions and land use.

Although not always counted in environmental food studies, post-production includes waste. Globally, about one third of food produced is discarded per year [36] with enormous global warming potential [37]. Food loss can occur along the entire supply chain, from harvest to consumer-level discards. The amount of food waste is generally higher in developed countries, although developing nations also show food loss, especially during production and harvest [38]. In developed nations, consumer-level food waste (e.g., households) is significant [39]. In the US, food waste from households has increased by 50% since the 1970s [40]. On average, 40% of food in the US is wasted each year [41]. This amounts to 9 kg of food wasted per person per month or 200 kg of food per 4-person household per year. This has been estimated to cost the American family at least $589 and the entire country $165 billion per year [42]. Food waste is a significant contributor to resource depletion, considering energy, water, and land are needed for production, distribution, and storage of the food that goes uneaten. Moreover, discarding the food adds a further burden to the environment, accounting for 25% of landfill-generated CH_4 [41]. Thus, besides the energy-costly inputs and GhG emissions from food produced that is unconsumed, wasting it contributes to environmental degradation.

Finally, a significant impact of agriculture on the environment is also its water use. About 70% of all surface and ground water goes to agriculture, with many aquifers showing diminishing reserves [43]. As water resources are becoming equally scarce as land, it is important to consider the significantly greater water footprint of beef production as compared with alternative meat and plant sources [19,21, 44], although there are some exceptions [13].

Studies that focus on food and the environment use Life Cycle Assessment (LCA) to quantify global warming potential of the entire food supply chain—from cradle to grave, including all resources used and all emissions to air, soil, and water. While GhG emissions specific to agriculture are commonly reported, comprehensive LCA studies also include land and water use, toxicity to ecosystems and human health, biodiversity loss, eutrophication, and ocean acidification [45]. Although beyond the scope of this paper, the reader is encouraged to consult further literature on this topic [7,13,14,46,47].

1.2. Dietary Change to Reduce Environmental Impact

Studies have shown that dietary change can play a significant role in reducing the impact of agriculture on global warming potential, land and water use. Recently, scientists have also linked environmental impact, nutrition, and health in the discourse of dietary change [13,48–50]. When considering dietary change as a realistic pathway for the reduction in GhG emissions, land and water use, one of the simplest approaches is to follow healthy dietary guidelines, including a reduction in calories [50,51]. This should not be underestimated since reducing calories, especially if achieved by increasing fruit, vegetables, and dietary fiber at the expense of meat, would result in weight loss and improved health, with enormous impacts on society, including health care cost [51–55].

Animal agriculture is the most costly for the environment [19,21,52]. Eshel et al. 2014 have demonstrated that ruminant (beef) production requires 28 and 11 times more land and water and emits 5 times more GhG, compared with the production of non-ruminant protein sources (e.g., chicken, pork, eggs). Converted to food and protein, beef has a 35:1 feed-to-food caloric ratio compared with a 10:1 ratio for other animal proteins and an 800:1 ratio for feed calories-to-protein ratio, which is almost 10 times lower for other animal protein sources [19]. Thus, eating less beef is becoming an important dietary message worldwide [56]. However, there can be even greater reductions by lowering meat consumption in general [19], and replacing meat, and especially ruminant meat, with plant-based alternatives, which reduces land, energy, and water use, while lowering GhG emissions and waste [21]. While dairy is more efficient than beef, emitting less GhGs, dairy production exceeds egg, poultry, and pork production in land and water use [19,53,57]. Thus, dairy production also contributes to the expansion of cropland and resource extraction which, together with beef production, eventually exceeds the Earth's safe operating space [58].

Reducing beef consumption and replacing some with plant-based sources, chicken, pork, or eggs could decrease GhG emissions by up to 35% from the food sector [35]; however, a moderate reduction and replacing beef with dairy has a negligible effect [50]. Replacing beef with fish may provide some benefit but this largely depends on the type of fish, its production system, and fish feed used in aquacultures [3]. Eating less beef can reduce land use by 50% to 70% (see reviews by Hallström et al., 2015 and Aleksandrowicz et al., 2016 [50,59]). Thus, consuming less beef (and dairy) could slow land clearing for feed production and some of this land could be repurposed to grow food for human consumption [53].

Eating less animal and more plant protein in general is also in line with governmental dietary guidelines [56], since most developed nations exceed protein, and especially meat, recommendations [3]. A recent article entitled "Protein production: planet, profit, plus people?" recommends people eat one third less protein overall, replace one further third of their protein intake by plants such as beans, nuts, and grains, and choose the final third from free-range animals [60]. Based on annual per capita intake data [61], if this rule were applied to meat intake, this would still give the average American 80–100 grams (3–4 ounces, oz) per day.

Considering dietary change that could contribute to climate change mitigation, shifting from a typical Western diet to a more environmentally sustainable diet with less meat and more plants would work [59]. Being vegetarian or vegan would be better, with over 30% and up to 70% reduction potential in GhG emissions and land use [21,59,62] and 50% less water use [59]. However, vegetarian or vegan lifestyles may not be preferred for many people [63]. In addition, recent advances also point toward beneficial roles of well-managed, sustainable grazing practices that promote carbon sequestration on rangelands [64], and some areas in the world are less suited for crop production but still provide a great place for livestock, including ruminants. Adding more value to the consumption of meat is necessary [60], however. Thus, in the above example by Aiking (2014), the last third of what used to make up a meat-based dish, should contain a source that can be traced back to its origin, showing a healthy environment where animals are part of an intact ecosystem, given a good life and an end with dignity [60].

2. Dietary Guidelines and Sustainability

It is quite clear that eating less meat (especially less red and processed meat), besides eating less overall and more whole and plant-based foods (i.e., vegetables, fruit, nuts, beans, grains), would be one of the most important dietary strategies for both planet and people. These recommendations are also grounded in the dietary guidelines of many countries, some of which have integrated sustainability [56].

In recent years, governmental dietary recommendations from various countries have begun to integrate sustainability. According to a recent report by the Food and Agriculture Organization (FAO) [56], of 83 countries that have official dietary guidelines, there are 4 reported countries that reference environmental factors in their dietary guidelines. These include Sweden, Germany, Brazil, and Qatar. Table 2 highlights sustainability commitments beyond those generally targeted to health (e.g., increase plant foods).

Table 2. Sustainability commitments in Germany, Brazil, Sweden, and Qatar.

	Germany	Brazil	Sweden	Qatar
Sustainability Highlights	Eat meat in moderation. Use fresh ingredients. Take your time and enjoy eating. Eat fish once or twice a week.	Choose seasonally and locally grown produce. Try to restrict the amount of red meat. Limit the amount of processed foods. Eat in company. Develop, exercise and share cooking skills. Plan your time and make food and eating important in your life.	Eat less red and processed meat (no more than 500 g of cooked meat per week). Choose eco-labelled seafood. Try to maintain energy balance by eating just the right amount.	Limit red meat to 500 g per week. Avoid processed meat. Eat less fast foods and processed foods. Build and model healthy patterns for your family. Eat at least one meal together daily with family.

The first country world-wide to awaken awareness regarding sustainability and food consumption was Sweden in 2009, calling for a reduction in meat in consumers [65], with a cohort of countries today advising to reduce overall meat consumption to 500 g per week (16–17 oz) [56]. Current meat intake in the US is almost 4 kg (9 pounds, lbs) of trimmed, boneless meat per week, with an annual per capita consumption of almost 90 kg (195 lbs) [66]. However, the US is not alone, as many European and South American countries, along with Australia are also high, but not quite as high. Calculating the yearly per capita consumption per sustainability guidelines, with 500 g per week or a total of 26 kg annually, this equates to about one third of the current US consumption pattern.

While inclusion of sustainability into the US Dietary Guidelines would have been highly significant, considering (1) the high calorie and meat consumption in the US and (2) the potential global impact of US dietary guidelines [67], it remained invisible in the official guidelines [68]. Although not part of national dietary advice, several countries have published scientific papers that focus on mathematical modeling to derive a regional, sustainable and healthy diet alternative to what is considered the norm. The New Nordic Diet and the Low Lands Diet are two such examples. Both studies focused on less meat, more (Nordic Diet) or less (Low Lands Diet) fish, and local, traditional foods, and both used the Mediterranean diet as benchmark to link health and sustainability [69,70]. Further, there are several quasi-official guidelines, from government agencies or government-funded entities that also include sustainability [56]. Most recently, the Netherlands published an update through the Netherlands Nutrition Centre, calling its citizens to action to reduce red meat intake to less than 300 g per week, while the UK's governmental agency, encompassing England, Wales, Scotland, and Ireland also added a 7% reduction of dairy products [71].

2.1. Are People Willing to Change Diets to Protect the Environment?

When dietary guidelines promote a change, press releases are often the next step, communicating the governmental messages to the public. However, it is well known that dietary guidelines are only marginally followed [72] and that eating behaviors are difficult to change [63], especially if guidelines remain a verbal or written recommendation without the practical skill building required to

put the guidelines into practice [73]. In addition, simply telling people what they should eat without communicating the reason behind this recommendation or focusing too much on diet and health may not work either. For example, Hekler and colleagues (2010) showed that a college course on society, ethics, and food changed eating practices more favorably than in students taking courses in health with a focus on biology, obesity, psychology, or community [74]. However, what about sustainability and the environment? Do people (1) understand the link between eating less meat and climate change and (2) would they make the change if it were both good for health and good for the planet?

"Eat as if there is no tomorrow" [63] studied a sample of Scottish people living in rural and urban areas using focus groups and interviews. The purpose of the study was to examine the perceptions of people toward eating less meat. The authors identified the following common themes, using a qualitative analysis: there was (1) a general lack of awareness related to the link between climate change and meat consumption and (2) little understanding that personal choice regarding meat consumption had anything to do with climate change. Finally, the study also showed that those interviewed were generally resistant to reducing meat intake.

Meat is a traditional menu ingredient in many cultures—meat is often the center piece of the plate. It should be apparent that dietary behavior change will only occur if people begin to understand how best to reduce meat intake. This requires innovative menu design, similar to what has been proposed by the Culinary Institute of America (CIA) and Harvard School of Public Health with the Menus of Change initiative [75], in addition to public campaigns such as Meatless Mondays [76]. This was also the synthesis of a recent study [73], proposing that besides policy change, innovative culinary training through reskilling to cook more balanced vegetarian meals would be necessary. In other words, food literacy training will be needed to bring these ideas closer to consumers.

Regardless of approach taken, promoting meat reduction for personal and planetary health, may continue to be challenging, as was shown by de Boer, de Witt, and Aiking, 2016. These authors studied people's perceptions as to the extent to which personal dietary change could mitigate climate change [55]. Few recognized eating less meat as an effective way to mitigate climate change, but those who did, showed greater willingness to eat less meat. When asked to rate personal preference of (1) eating less meat; (2) eating more organic food; and (3) eating more local/seasonal food as a vehicle to mitigate climate change, eating more locally/seasonally grown food appealed to more individuals than the other two, including the message of eating less meat [55].

2.2. Duality of Sustainability and Health

As we begin to imagine how to integrate sustainability into healthy and athletic lifestyles through the food we chose, we must define what constitutes sustainable food. Sustainability means that "humanity has the ability to make development sustainable—to ensure that it meets the needs of the present without compromising the ability of future generations to meet their own needs" [77] (p. 5). The three pillars of sustainability which include equity, environment, and economics often focus on what is currently unsustainable, for example in food production, but also tend not to challenge the consumer in moving to sustainable development. Sustainability is a moving target, dynamic and ever changing, as the planet is changing. Thus, adapting with the goal to mitigate climate change is needed in all sectors of production, distribution, consumption, and resource recovery, globally as well as locally. While there are numerous examples of sustainable agricultural advances, including organic production [78] and perennial polycultures [79], promoting greater social equity for farmers and welfare for animals and focusing on sustainable consumption patterns are also important. So, what is a sustainable diet? "A sustainable diet is a diet with low environmental impacts which contributes to food and nutrition security and to a healthy life for present and future generations. A sustainable food system is protective and respectful of biodiversity and ecosystems, culturally acceptable, accessible, economically fair and affordable, nutritionally adequate, safe and healthy, while optimizing natural and human resources" [80] (p. 111).

In 2014, Kjærgård and colleagues published a framework on how to link sustainability and health and by doing so, dual benefits could be observed [81]. These authors referred to their concept as a duality between sustainability and health. Considering sustainable food choices, a sustainable diet would also be a healthy diet. To understand this duality concept a bit deeper, the example of meat shows the duality of sustainability and health quite well. In general, livestock production, especially beef production, is associated with resource depletion and pollution [4] and contributes significantly to GhG emissions, biodiversity loss, and high health care costs [2]. Excessive red meat consumption has been associated with poor health outcomes, such as cardiovascular disease, obesity, diabetes, and cancer [5,7,82,83]. In addition, how animals are raised in some countries, in confined animal feeding operations (CAFOs), is also of concern regarding animal and worker welfare in the context of community prosperity, health, and quality of life near CAFOs, and human antibiotic resistance from non-therapeutic use of antibiotics in such production systems [84]. The duality of health and sustainability shows that addressing the "eat less meat" message has co-benefits for both sustainability and health. From the consumer side, dietary approaches that promote high protein intakes, such as the westernized diets and those recently termed "the diets of the healthy and wealthy" [85] fail to consider this duality approach, and thus, slow the shift urgently needed to promote both sustainable and healthful eating. There are other examples that provide insight into the intertwined synergies between sustainability and health, such as the ecosystem and health services arising from urban farms and community gardens [86] as well as reducing food waste [87].

Taken together, the inclusion of sustainability in nutrition and health is critical for current and future generations. The science of nutrition must embrace environmental considerations similar to the time when public health nutrition emerged from the science of nutrition for individuals and expanded its reach to communities [54]. "The nutrition of individuals and communities can only be maintained within an environmentally sustainable context, which is currently under serious threat." [54] (p. 817). Thus, we are in the center of a difficult reality—a sustainable food system needs the support of an intact ecosystem, but the way we currently eat contributes directly to its degradation [54].

3. Eat as If You Could Save the Planet and Win!

Whether the intention is health, fitness, and/or sports performance, integrating environmental consciousness when making dietary choices, seems no longer an option but rather a necessity. And while global warming and climate change are often overwhelming topics, dreaming the alternative fires up the imagination of young people, as so beautifully described in the Future of Health by Hanlon and colleagues [88]. Thus, to end a long discourse on the unsustainability of the current food system and its apparent lack to teach us anything about good food and healthy communities or environmental conservation, while the alternative does, the next section will focus on how to integrate these ideas into the daily eating practices of those who train to win.

3.1. Ecological Footprint: This Gets us Thinking

To understand one's own impact on the environment, it is always a good exercise to calculate the ecological footprint [89]. This is especially true with respect to dietary choices, as most people do not make the connection between their own eating and climate change [63]. However, there are also tradeoffs. Some might travel a lot by plane, which increases one's footprint significantly. A good tradeoff would be to make sustainable dietary choices to contribute to environmental protection.

3.2. Sustainability in Sports Nutrition?

The integration of environmental nutrition concepts might not be as intuitive in sports nutrition but there are many entry points. One might also argue that due to high energy intakes to meet energy demand, enormous use of packaged foods and bottled beverages, equipment, materials, and heavy travel schedules athletes and their teams should integrate sustainable practices whenever possible.

From a health perspective, athletes lead a more sustainable lifestyle than most of society. Athletes rarely burden the health care system due to chronic disease such as obesity and diabetes. And participating in sports should play a substantial role in making sustainable healthy lifestyle choices. Athletes are also great icons for kids to pick up sports. Athletes are role models for society at large and are generally represented by values of good sportsmanship [90]. Athletes are also great spokespeople, sharing their lessons learned through sports (e.g., time management, discipline to work hard, the importance of rituals, discerning the meaning of failure or injuries). While still dormant, athletes could become a strong voice for planetary health, and begin to realize that success in sport depends, in part, on an intact food system. Athlete or non-athlete, all young people should receive sustainability literacy training, and a covert approach to nutrition education may work best, using experiential learning through taste education, farm visits, cooking and eating together. The conversation around the digital-free table can further the understanding of contemporary food topics and build knowledge surrounding the current issues of the food system and what the sustainable, tasty alternative is all about.

Finally, the sustainable diet is not only the athlete's responsibility. As we see with other big topics in sport nutrition such as eating disorders [91,92], if coaches, service providers, and administrators are supporting the underlying rationale of a refreshed approach to nutrition education, using sustainability principles, it will enable change. Athletes, coaches, service providers, and administrators all serve as role models for societal change, thus, the adoption of sustainability principles, while coming from the bottom up in our examples, are best diffused if top to bottom is committed and understands the rationale behind the effort. True sport needs true food!

Therefore, let's get athletes on board in saving the planet and winning! Because sport nutrition is often focused on quantity, quality, and timing of food intake relative to training and competition, below we list sustainability actions for these overall themes, add a section about food literacy and food citizenship for athletes, and consider some final thoughts regarding the integration of sustainable practices for teams, institutions, and international events, such as the Olympic Games.

We will focus on several areas that may apply to exercisers and fitness enthusiasts in general, but athletes in particular, making small steps toward a more sustainable diet as the overarching goal, and this begins with the work of the sport nutrition professional.

3.3. Quantity of Food

3.3.1. Eat Less and Better Meat

Athletes' diets are generally high enough in protein [93,94], if not excessive [95,96]. Current protein recommendations have increased for athletes [97], ranging from 1.2–2 g/kg body weight (BW)/day, especially if the goal is muscle protein accretion [98]. Recently, these recommendations were translated into practical strategies to help athletes maintain protein consumption at intervals throughout the day in the amounts of 0.25–0.3 g/kg BW [99] or about 20 g [100] per meal, given several eating occasions (best every 4 h) [101] per day and before sleeping [102]. This has also been summarized in the recently released position paper on Nutrition and Athletic Performance [97]. That athletes follow guidelines for protein intake is shown in the most recent dietary study on a sample of well-trained Dutch athletes, with mean daily protein intakes of 108 $\pm$ 33 g (1.5 $\pm$ 04 g/kg BW/day) and 90 $\pm$ 24 g (1.4 $\pm$ 0.4 g/kg BW/day) in men and women, respectively [94].

There have also been recent trends for even higher protein recommendations to promote health [103], support weight loss strategies [104], to preserve lean body mass (LBM) under hypocaloric situations [105], in resistance-type sports such as bodybuilding [106], and corporate sports performance programs [107]. That athletes, especially in strength and power sports, accomplish higher protein intakes has also been shown [108], with recent reports also highlighting the issue of extremely high protein intakes in some athletes [96,109]. Even though data are limited, practitioners should be well aware of excessive protein intakes in some athletes, aligning with current sport nutrition trends,

including the paleo diet. Finally, practitioners may inadvertently promote high protein intakes, considering educational tools and strategies or athletes may simply get too much by eating a lot, since protein is a function of energy intake [110]. However, what are the concerns besides the fact that some athletes may overdo it without proper guidance?

Because this paper is about sustainable diets, the question arises if current protein recommendations for athletes and actual intakes are going to align with global recommendations to reduce rather than increase protein intake in developed countries. Meeting protein recommendations in athletes per se may not necessarily be the issue. The issue is that the continued emphasis on higher protein needs will likely increase the demand for animal protein, including meat, dairy, and eggs. Considering the 50% rise in the world's population since 2000 and society's insatiable hunger for meat, the world meat and cheese demand will double by 2050, further burdening the planet [4]. Animal proteins are already consumed in greater quantities than plant proteins, in both the general [66,111] and athletic population [94], and the US considerably exceeds European countries in daily animal protein consumption [112].

Table 3 shows hypothetical amounts of meat (in this case beef) in reference to the (1) non-athlete recommended daily allowance (RDA) for protein [113]; (2) current athlete protein recommendation (~1.5 g/kg BW/day [97]); and (3) recently suggested athlete protein recommendations under energy restriction for weight loss (~2.5 g/kg BW/day [104,105]). It is assumed under this example, that 50% of dietary protein is supplied by meat. This is a rather conservative estimate based on total animal protein intakes typically exceeding 65% in the general population [111].

Table 3. Daily protein recommendations using estimated daily meat contributions for athletes and non-athletes.

Example	Units	Non-Athlete PRO RDA	Athlete's Standard PRO	Athlete's Hypocaloric PRO
60 kg female	PRO (g/day)	48	90	150
	Cooked Meat Contribution as 50% of total PRO (g/day) *	92	172	288
80 kg male	PRO (g/day)	64	120	200
	Cooked Meat Contribution as 50% of total PRO (g/day) *	123	230	387

* meat contribution at 50% of total protein recommendation, calculated for cooked ground lean beef (15% fat); 100 g edible portion equals 26 g of protein (similar for chicken, pork, lamb). Athlete's standard diet calculated at protein recommendation of 1.5 g/kg/day [97]. Athlete's hypocaloric diet calculated at protein recommendation of 2.5 g/kg/day [104,105]. PRO = Protein. Most sustainable and healthy dietary recommendations target 300 g of red meat or 500 g of total meat per week [56]. RDA = Recommended Daily Allowance. Table shows how easily athletes may exceed these weekly meat recommendations if they ate 50% meat of the total protein recommended per day.

From Table 3, we can see that meat consumption may easily exceed what is currently considered sustainable, as a total of 500 grams (17.6 oz) of meat per week (~70 grams per day; 2.5 oz) and less or equal to 300 grams (10.6 oz) of red meat per week (~45 grams per day; 1.6 oz/day) would be the upper limit per person. These are also the upper limits for meat consumption of most countries' dietary guidelines [56], including those for Americans, to promote health [68].

If the recommendation by Aiking (2014) could be implemented it would mean to cut 1/3 of the protein (in this case we would focus on meat, especially red meat), replace 1/3 with plant protein (beans including soy, grain, nuts, seeds), and to choose grass-fed or pasture-raised animal protein sources to obtain higher quality meat with greater omega 3 fatty acids and antioxidants, [114], not to mention less agricultural chemicals and antibiotic residues [60].

Let's look at an example integrating the recommendation by Aiking (2014) from above [60] but with focus on meat, especially red meat. If an 80-kg heavy male athlete eats 120 grams of protein of which 50% comes from meat, it equals approximately 240 grams of cooked meat per day. This is

more than 3 times the 70-gram daily benchmark. Thus, if the athlete follows the recommendation for an environmentally friendly protein intake by Aiking (2014), they would first reduce this amount by 80 grams of meat which equals approximately 20 grams of protein [60]. Second, the athlete would creatively adapt protein intake, according to the Protein Flip Initiative (see Table 4), and replace another 80 grams (or 20 grams of protein) by plant sources (See Table 5). The question that will arise is whether the athlete should replace the first third of meat that was cut out, and if so, how would this be done within sustainable boundaries? The answer may be substituting red meat with chicken, pork, or eggs, or choosing a greater proportion of plant-based proteins (e.g., beans, peas, nuts, seeds, and/or grains). However, plant protein may lack essential amino acids (EAA), and thus, may be required in greater amounts to meet the RDA. A recent study compared the land use change and GhG emissions of various animal and plant sources in amounts corresponding to the RDA for EAA [115]. Interestingly, environmental impacts were no longer as discriminatory for animal versus plant proteins, with exception of soy, which showed the lowest GhG emissions and land use. However, we should be cautious when interpreting these data, because people eat a variety of foods in variable amounts to meet daily protein and EAA needs. According to the American Academy of Nutrition and Dietetics Position Paper on Vegetarian Nutrition [116], it is not necessary to get all EAA at one meal, and especially not from one plant or animal. Rather, EAA are accumulated over the course of a day from various foods, and it is not uncommon to find vegetarian meals enhanced with small amounts of animal protein (e.g., dairy, eggs), while vegan meals may include various protein-rich plant foods. Thus, the key message for omnivores is to reduce total amount of animal sources of protein, while for vegans, the message may be to ensure diets meet daily EAA needs by eating sufficient amounts of food, along with a combination of protein-rich, plant-based sources. Working toward a more balanced approach between animal and plant proteins should be the primary goal for both planetary and personal health. Considering the higher protein needs in athletes [97], bugs may be the most suited protein to make up the difference from non-athletic controls, however, at substantially lower environmental cost.

3.3.2. Insects

Insects may well be the next protein source with which excessive meat may need to be replaced. Insects are nutritious, with similar amounts of protein compared to livestock and high levels of vitamins and minerals. Insects can also be a good source of essential fatty acids. Insects emit much lower GhG due to their highly efficient feed-to-protein conversion rates and insects have very low water requirement [117]. Insect powder may become a viable option for post-exercise recovery nutrition in liquid or solid food products, some of which are already on the market. In addition, plant-protein alternatives, such as pea (*pisum sativum*) protein powder, may also present a carbon-friendly source for athletes [118]. Obviously, much more research is needed to compare various plant protein alternatives and insects to the well-researched and popularly used dairy proteins post-exercise. So, what about dairy?

3.3.3. Dairy

Milk, yogurt, Greek yogurt, and cheese all add up quickly, and most Americans, including athletes, may indeed meet the US dietary guidelines, recommending 700 mL of dairy products per day [61]. While milk consumption has gradually decreased over the past decades, cheese, yogurt, and whey intakes have dramatically increased [61]. It is estimated that milk production contributes 2.7% to total GhG emissions [119], although there is great variability based on farming systems [119], with industrial systems generally showing lower GhG emissions due to higher feed digestibility and milk productivity per unit of product, compared to extensive farming systems. However, if other components of environmental degradation (e.g., pollution of waterways and biodiversity), increased energy demand, and human and animal welfare—basically the sustainability of dairy production—are questioned [120], the impact of intensification may well be greater [119].

Table 4. Examples for protein flip menus and burgers.

Meal	Actual	PRO g	Protein Flip	PRO g	Comments
Grilled Beef with Quinoa and Veggies	4 oz beef	26	2 oz 100% grassfed beef	13	Rename to Southwest Anasazi Bean and Beef Bowl.
United States Olympic Committee	4 oz kale and quinoa	4	4 oz kale and quinoa	4	Launch educational campaign on protein flip.
Colorado Springs	4 oz broccoli	3	2 oz Anasazi beans	10	Add history of Colorado beans and quinoa.
	1/2 stuffed portobello	5	4 oz broccoli	4	
			1/2 stuffed portobello	5	
	total	38	total	36	
Pork loin with Poblano Chili and Rice	4 oz pork loin	26	2 oz organic pork loin	13	Rename to Ancient Grains with Poblano Chili Pork.
United States Olympic Committee	4 oz poblano chili	3	4 oz poblano chili	3	Launch educational campaign on protein flip.
Colorado Springs	4 oz white rice with veg	4	6 oz farro, beans, veggies	12	Integrate nutritional benefits of ancient grains.
					Add history of emmer and biodiversity of grains.
	total	33	total	30	
SWELL Burger	4 oz beef burger	22	2 oz 100% grassfed beef	10	This meal is served at UCCS Food Next Door.
University of Colorado	white bun	5	1.75 tsp black beans	2	SWELL Burger uses the protein flip approach.
Colorado Springs	1 cup dinner salad	1	1.75 tsp quinoa	1	Launch educational campaign on protein flip.
			1.75 tsp hemp	3	Integrate sustainable food literacy.
			1 T peppers, carrots, leeks, chard	1	Highlight nutritional benefits of grassfed beef.
			garlic, chili, cumin, chives		Include social justice issues regarding CAFO.
			1 slice socca (chick pea flatbread)	4	Highlight Slow Meat and Menus of Change ideas.
			SWELL kale salad with roasted veg	2	
			pumpkin seeds	2	
	total	28	total	25	

SWELL: Sustainability, Wellness, & Learning; UCCS: University of Colorado, Colorado Springs; PRO: Protein; CAFO: Confined Animal Feeding Operation; ounces (oz; 1 oz = 28.4 g); tsp: teaspoon; T: tablespoon.

Table 5. Cooked amounts of plant and animal-based foods delivering 20 g of protein.

Food	Grams	Ounces	Cups	T	Calories	Limiting Amino Acids	Leucine (g)
Anasazi Beans	322	11.4	1.4	23	426	Sulfur containing AA	1.2
Black Beans	295	10.4	1.3	21	295	Sulfur containing AA	1.3
Chickpeas	284	10	1.3	20	336	Sulfur containing AA	1
Soybeans	204	7.2	1	14	268	Complete plant protein	2.3
Lentils	250	8.8	1.1	18	253	Sulfur containing AA	1.3
Tofu	284	10	1.3	20	189	Complete plant protein	1.3
Tempeh	306	10.8	1.4	22	265	Complete plant protein	2.4
Edamame	318	11.2	1.4	22	265	Complete plant protein	1.2
Seitan	408	14.4	1.8	29	270	Complete plant protein	no data
Buckwheat	755	26.6	3.3	53	516	Complete plant protein	0.4
Quinoa	567	20	2.5	40	555	Complete plant protein	0.5
Millet	748	26.4	3.3	53	683	Lysine, threonine	0.8
Amaranth	500	17.6	2.2	35	552	Complete plant protein	no data
Einkorn	145	5.1	0.6	10	218	no data	no data
Emmer	227	8	1	16	200	Lysine	0.3
Spelt	411	14.5	1.8	29	445	No data	no data
Kamut	411	14.5	1.8	29	454	Lysine	0.8
Almonds	227	8	1	16	575	Methionine, Cysteine	2.1
Peanut butter	68	2.4	0.3	5	470	Methionine, Cysteine	3.9
Hemp seeds	57	2	0.3	4	160	Lysine	0.7
Pumpkin seeds	132	4.6	0.6	9	433	Complete plant protein	3
Beef 15% fat	73	2.4	0.3	5	157	Complete protein	1.7
Chicken	91	3.2	0.4	6	100	Complete protein	3.3
Pork	73	2.4	0.3	5	152	Complete protein	1.9
Milk 2% fat	567	20.0	2.5	40	284	Complete protein	0.8
Eggs	188	6.4	0.8	13	291	Complete protein	2
Fish (tuna)	141	4.8	0.6	10	179	Complete protein	3.2

T: tablespoon. Combining protein-rich, plant-based foods will be the best strategy in obtaining all amino acids if partially or fully replacing animal-based foods.

Globally, about 45%, 20%, and 35% of milk is processed into cheese, milk powders, and fresh or fermented dairy products, respectively [119]. In the US, 50% of raw milk is generally processed into cheese [121]. Milk production generates about 1 kg of CO_2 eq/kg of milk (or 2.4 CO_2 eq/kg ready to consume milk) at farm gate [119]. Additional processing, transport, and distribution for dairy products, such as cheese, whey and yogurt increase GhG emissions [119]. Finished products, such as cheese and yogurt, show greater emissions due to the fact they need more milk per unit produced (see Table 1).

Depending on current dairy intake, a climate friendly start could be to reduce dairy products in general, and cheese in particular, due to greater GhG emissions [35]. The UK [122] currently suggests a 7% decrease in dairy, among reductions in meat, for all citizens to participate in consumer-driven climate change mitigation. This is world-wide the only guideline that targets reductions in dairy. Athletes may want to focus on milk, rich in whey, in the recovery period after an important workout, since this is an effective protocol to promote post-exercise protein synthesis [97] and is palatable. Whether environmental differences exist among milk-derived protein depends on what functional unit is used to express GhG emissions. A Canadian study shows that per gram of protein, GhG emissions are similar or slightly less for cheese and yogurt compared with milk. However, per kg product, milk ranks significantly lower in GhG emissions than cheese and yogurt [123]. Should an athlete need to focus on extra weight/muscle gain, casein-rich Greek yogurts appear popular before going to bed to promote protein synthesis at night [102]; however, Greek yogurt emits more GhGs than regular yogurt, because its production requires more milk [121].

While sweetened yogurts are often loaded with sugar and unrecognizable ingredients, a good choice is the least processed type that contains naturally occurring beneficial bacteria from

fermentation. These bacteria are generally known as probiotics and are thought to boost gut health [124]. Thus, for both the environment and health, less processing in yogurts may be the way to go. Because most of the sport nutrition research has been conducted using dairy products, future studies are needed on more environmentally conscious plant protein alternatives and insect protein. This is especially important for athletes who, by default, likely exceed animal protein recommendations from meat and dairy (including whey), currently deemed unsuitable to protect the environment.

3.3.4. Reinventing the Athlete's Plate

To make the message of meat (and dairy) reduction palatable, practically engaging initiatives are needed. Choosing less and better meat is Slow Food's global strategy [125] for developed nations, where meat intake is generally very high. Flipping protein on the plate and making meat the topping or side dish is a strategy promoted through the Culinary Institute of America's Protein Flip initiative [75,126], which originated from the Menus of Change collaborative between the CIA and Harvard School of Public Health. Recreating the plate using meat as a garnish and complementing this dish with whole grain pasta, potatoes, vegetables, and protein-rich grain, legumes, nuts, and seeds is also an easy and creative way to rebuild an athlete's plate. This is the current topic of ongoing research at the United States Olympic Committee's (USOC) Food and Nutrition Services, as the Athlete's Plate [127] was shown to promote more protein than recommended for easy, moderate, and hard training days [128]. Further analysis indicates that the protein dished up on the plates by trained professionals was mostly of animal origin (more than 70%) with marginal amounts of plant protein [129]. It is expected, as was previously shown [73], that food service organization and restaurants may lack the experience with meat-reduced, vegan and vegetarian cuisine. Thus, while flipping proteins of animal-based plates is becoming more popular, taking a closer look at vegan and vegetarian menus and their composition will also help promote plant-based meals for omnivores. Once culinary professionals, students, and nutrition professionals tackle such menus, calculating nutrient profiles could be helpful [130,131], as the outcome of a protein flip menu should not compromise nutrient density—in fact, it should improve it. Most athletes consume sufficient calories to meet micronutrient needs and the majority also takes dietary supplements [132] and eats fortified foods (e.g., cereals, bars), which makes the integration of plant-based eating less concerning. Our preliminary work with the USOC Food and Nutrition Services shows hypothetically that (1) protein flip menus (with less meat) and (2) improved vegetarian menus, increase rather than compromise nutrients, while protein remains at moderate yet recommended levels for athletes [133]. The University of Colorado, Colorado Springs, having transitioned from a corporate to a self-operated dining and hospitality system, recently adopted the CIA's Menus of Change initiative and serves a very popular protein flip burger at its local food station called "Food Next Door" [134] (see Table 4 for examples).

Protein flip and vegetarian menus provide greater amounts of carbohydrate and fiber [116]. While extra carbohydrates are performance-enhancing, there may be concerns that phytates from fiber may inhibit iron absorption, thus, making the iron from meat less available. One strategy to assist with improving bioavailability of these changed menus is through iron enhancers, including fermented foods. Lactic fermentation is one of the oldest methods for food preservation [135]. Research shows that lactic fermentation of vegetables, corn, and soybeans can drastically reduce phytate content [136], thereby reducing its effect on nutrient absorption. For iron absorption, the mechanism is thought to be through the increase in ferric iron (Fe^{3+}), enhancing iron bioavailability [135]. It has also been shown that fermented sauerkraut improves iron absorption [137] and that fermented foods contribute to enhanced nutrient bioavailability in Asian cultures [138].

3.3.5. An Omnivore's Choice to Eat Vegan

While vegan diets may need more caution to ensure protein quantity, quality, and complementarity as well as achieving athletes' energy availability, it is generally accepted that these diets do not present with adverse health [116] or performance effects [139–142]. In fact, most data show that

plant-based diets are not only great for the environment [13,50], but also human health [13,116,142], and they may promote performance enhancement [123,143]. While some athletes may use vegan diets to mask an eating disorder, there is no evidence that vegan or vegetarian diets cause eating disorders [144]. Considering that a reduction in meat, using more plant-based approaches, is effective in decreasing environmental impact does not mean that athletes must turn vegan. However, integrating meat-less meals and days in omnivorous athletes is not only fun and healthful but it is also educational. Making tasty and nutritionally-balanced vegan meals can also mean a new challenge for those in the kitchen. If proper screening and assessment of individual athlete risk precedes the introduction of plant-based dietary approaches, and education is provided about the rationale for such an approach, there should be no concern.

The best start into an environmentally friendlier diet for athletes is to start right here. As sport nutrition professionals, we need to understand the impact diet has on the environment. Athletes can simply consider the total animal and plant protein contributions in their diet and aim to reduce (not eliminate) red meat first, followed by integration of more plant-based protein choices. A closer look at dairy protein may also be warranted. If everyone in the United States ate no meat just one day per week, it would account for the carbon equivalents of driving 91 billion miles less or taking 7.6 million cars off the road [145]. Reducing meat consumption, in general, can have significant savings overall in food-related GhG emissions and land-use change, exceeding what can be achieved from the transportation sector [50]. Recent research also highlights the individuality of diets and that reductions in environmental impact can be achieved using various approaches, not necessarily compromising personal, cultural, or economic factors [146]. While animal protein reductions in athletes should be of primary importance considering environmental conservation, overall protein intake, nutritional status and the athlete's cultural background will determine if this is the best approach to take. However, we should not forget to highlight athletes who have been using vegan and vegetarian approaches and athletes who stand up for a healthier environment and restorative farming practices [147].

3.4. Quality of Food

3.4.1. Plant Biodiversity—Diet Diversity

In the last 100 years, three quarters of plant and animal species globally have been lost, and the majority of the world's food supply comes from a dozen plant and a handful of animal species [80]. At the same time, food processing has increased in a way that creates an artificial diversity and a false sense of food security, when browsing through endless aisles in a grocery store. Perhaps it is this level of agricultural simplification that has made the broad field of nutrition oblivious to the topic of biodiversity. Balanced nutrition depends not only on a variety of foods in the diet. The human diet also depends on the diversity within a food crop [148]. While largely understudied and under-documented, fragmented data show vast differences in nutrients within the same species; for example, in potatoes, rice, mangoes, bananas [149,150], and tomatoes [151], but also indigenous corn grown in the American Southwest [152]. Perhaps one of the most striking results in nutrient density comes from the potato, a staple of many countries, and often marginalized as a processed fast food not tolerated on healthy plates. Potato biodiversity is still broad, with over 5000 known varieties remaining and vastly differing nutrient content [150], particularly for sugar, protein, potassium and vitamin C. Similarly, wild plants, still contributing significantly to the health-promoting properties of the Mediterranean regions, have higher amounts of vitamins A, C, and those of the B-complex compared to their cultivated counterparts [153]. Interestingly, wild and local foods are increasingly being recognized as an integral part of contemporary nutrition, as countries are redefining their dietary guidelines, linking sustainable and healthful eating in a traditional context [154,155]. Unfortunately, crop biodiversity and its role in nutrition is generally neglected and this may be due to the field's professionals [149]. Perhaps a visual comparison as shown in a recent New York Times

article [156] brings the message home. We simply assume that a tomato is a tomato and that nutrient density will remain the same despite significant differences [151]. It is true that nutrition education appears to be almost blind to biodiversity [80], although resources are available [157]. Diet biodiversity is becoming a rapidly emerging field but has remained understudied, especially in the nutrition sciences. However, with the return to the farm, scientists are recognizing that agricultural biodiversity can support food and diet diversity, thereby improving nutrition and health [158]. Research is also emerging that agricultural intensification, characteristic of high yield outputs, is associated with the loss of rare plant species [159], but shifting to more sustainable systems, biodiversity may be conserved and ecological functions secured [11]. Losing biodiversity means loss of diet quality, which can lead to micronutrient deficiencies, food insecurity, more pests on farms, fragile ecosystems, and the loss of culture and tradition. Thus, biodiversity should not only be recognized as an important player in sustainable agriculture, but also as a necessary contributor to a healthy diet [160].

3.4.2. Nutrient Composition and Nutrient Density

Dietary choice from the farm or factory gate produces variable foods with variable consequences. Meat and dairy from cows grazing on pastures all their lives provide a nutritionally superior [114], healthier, and safer product [25,52,84], especially considering antibiotic use in CAFOs [161,162]. However, grassfed beef is generally more expensive and considered less sustainable because more land is needed for animals to graze, with greater GhG emissions per kg of beef produced [18]. Unfortunately, animal welfare is not yet part of LCA studies, thus, intensive, as opposed to extensive farming systems, usually fare better in both GhG emissions and land use [18].

Considering the topic of fish, omega-3 fatty acids, especially eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found variably in fish, wild and farmed, have significant health benefits [163] recognized by health organizations world-wide [164]. Fish oils are also popular in athletes [165]. However, fish has been a topic of much debate, not only because of variable omega 3 fatty acid content but also environmental contaminants. Whether farmed fish contains lower, comparable, or greater amounts of EPA and DHA than a wild-caught counterpart continues to be an equivocal topic [166–169]. The type of feed (plant vs. fish-based) used in farmed fish is one important consideration [170]. Recent trends of vegetable oils in salmon feed have shown to increase the proportion of omega 6 fatty acids, while omega 3 fatty acids decrease [171], potentially impacting negatively on both fish and human health [172]. Although wild fish supply is diminishing fast, if people were to eat the wild fish that are seasonally available rather than the wild fish they desire (e.g., salmon), there would continue to be some level of access—at least for a little while [173]. However, wild fish supplies will not be able to meet the rising demand of a growing world population [3], nor will wild fish necessarily be free of pollutants [168]. Already to date, more than 50% of all fish consumed globally come from aquacultures [3]. Aquacultures generally emit lower GhG compared to wild fisheries, although concerns exist about the feed used in aquacultures [18]. While disconnecting marine resources from fish farming is recognized as an invaluable progress for protecting marine ecosystems, it does not come without increasing challenges about the feed used in aquacultures, especially if produced terrestrially [174]. Perhaps plant alternatives (e.g., microalgae) will be able to provide sustainable solutions in the future, so humans can continue to benefit from fish-derived EPA and DHA.

What about conventional versus organic production? Organic milk provides a superior nutritional profile than conventional milk, with organic milk containing more protein and omega 3 fatty acids [175], and raw milk may provide potential protection against allergies, although there is a greater risk of pathogens [176,177]. On a crop-level, nutrient composition in food has suffered in the last sixty years, with nutrient losses of up to 30%, most likely due to depletion of soil nutrient quality [178]. Conventional agriculture may produce food more economically and in higher quantities. However, research is gradually emerging, showing ecological and human health repercussions of such systems [19–21,25,26,29]. Organically grown soybeans show significantly higher nutrient composition, including amino acids, total protein and several micronutrients, compared to conventional and

genetically modified (GM) soybeans. While the debate continues whether organic vs. conventional produce is superior in nutrients [78,179], organic foods contain significantly less herbicides, pesticides, toxins, and antibiotic residues [179,180] compared with conventionally produced food. Organic systems can also be more energy-efficient, may thrive in drought conditions, tend not to pollute waterways with synthetic pesticides and nitrate, and typically protect ecosystem services, such as biodiversity (see Reganold and Wachter, 2016, for an excellent review [78]). Finally, while largely understudied considering nutrient content, local food systems provide the most direct pathway from farm to table, with potential for greater nutrient density due to seasonality and reduced transit time from farm to consumer [181].

Taken together, from animals to plants, we must begin to pay attention to the quality of food. In addition, people must understand that dietary choices have the power to either protect or degrade ecosystem and health services. This knowledge may be difficult to teach in a classroom, and it is not nearly as fun as going to the farm. Farm field trips may be especially health-promoting for young, active children, as recent studies show the immune benefit of growing up on the farm [182].

3.4.3. The Grain Chain

The discourse on better food for health of both planet and people, however, is not complete without a discussion on grains. In addition, grains remain the world's most important staple [80] and may be a key strategy of the Menus of Change initiative. And yet, there has not been more controversy regarding issues of modern wheat [183], including its higher amounts of triggering gluten proteins [184]. Gluten-free eating has seen a tremendous popularity. In athletes, studies show that over 40% of athletes adhere to a gluten-free diet even if they do not have to [185], and despite the fact such diets do not improve performance [186]. However, are all grains as evil as they sound?

First, whole grains are packed with fiber, protein, carbohydrate, and B-vitamins and studies show whole grains reduce all-cause mortality and morbidity, with lower risk for cardiovascular disease, cancer, and diabetes [187]. When wheat is grown organically it has been shown to contain superior nutritional profiles compared to conventional wheat [188].

Wheat's nutritional profile has significantly decreased since the 1960s [189–191], while the number of new gluten proteins has increased [192], and this is reportedly not due to changes in soil, but changes in wheat hybridization. On the other hand, ancient wheat such as einkorn, emmer, kamut, durum, and spelt, celebrating a recent comeback, exceed nutrient composition (e.g., protein, lipids, minerals and elements, antioxidants such as lutein) compared to modern wheat [190,193–195]. A recent study on khorosan, also known by the name Kamut, shows greater anti-inflammatory effects through antioxidants, blood minerals, and reduced metabolic (e.g., lipids, glucose) and oxidative stress markers in healthy subjects consuming khorosan in bread, pasta, and crackers for 8 weeks, compared to consuming these products made with a semi whole-wheat product [196]. Thus, these older cultivars may contribute significantly to nutrient dense diets [195] and health promotion [196].

Such ancient wheat varieties are not only more nutritious and promote antioxidant protection, but some also lack the highly immuno-suppressive α-gliadin peptides—the major component of gluten that provokes gluten intolerance. These are encoded by the D-genome of wheat. Thus, species that lack the D-genome of wheat, such as einkorn, emmer, and durum, show lower reactivity compared to common wheat [197]. Work in Italy is currently focused on einkorn, the oldest form of wild wheat first domesticated 12,000 years ago by hunter-gatherers in Mesopotamia. Along with wild emmer, also known as the mother of all wheats, "einkorn is considered a catalyst of agriculture and the initiation of wheat's vast biodiversity" [198] (p. 22). Einkorn appears to either pose no [199] or fewer adverse reactions in Celiac patients compared to modern wheat [200]. Athletes who have been diagnosed with Celiac's disease, should consult their sports dietitian before trying einkorn since it may still have the potential to induce the Celiac's disease syndrome [201]. For a great review see Kucek, Veenstra, Amnuaycheewa, and Sorrells, (2015) [202].

Understanding nutritional differences among grain varieties also opens the dialogue on bread. While the choice of grain permits greater nutrient intake, fermentation using a sourdough starter has also been shown to increase bioavailability of nutrients such as iron. This is most likely due to a reduction in phytates [135]. Fermented bread decreases post-prandial glycemic response through organic acids that delay gastric emptying [203]. This, therefore, is a great low glycemic alternative to processed white bread for active individuals, especially at breakfast. Finally, sourdough fermented bread also appears to retain antioxidants better due to lower pH levels [204], which if baked with an antioxidant and protein-rich grain, such as einkorn or emmer [193], by far exceeds the nutrient density compared to bread made with modern wheat.

Studies also show that both germination (sprouting) and fermentation (e.g., sourdough baking) can break down gliadin, one of the gluten proteins known to increase reactivity. While still not safe for Celiac patients [205], there are fewer immunoreactive peptides in sprouted products [202]. As for fermentation, lactic acid bacteria degrade some of the gliadins but multiple microbes appear to be needed to effectively degrade the majority of gliadin [206]. In a study by Greco et al., (2011), 97% of gluten was degraded by fermentation [207]. However, there were still a few Celiac patients in the study who showed measurable villi atrophy compared to non-gluten control treatments. Thus, wheat sourdough fermentation, as compared to non-fermented flour, does not degrade gluten enough to prevent adverse responses in Celiac patients [208].

Taken together, there does not seem a clear relief for Celiac patients, from ancient or heritage wheat, whether sprouted, fermented, or not. However, research suggests that there may be wide variability among reactivity to gluten, depending on the type of grain and level of processing. In addition, Celiac's disease expression, while triggered by gliadin-induced antibodies, can also be quite variable. While Celiac's disease has become more prevalent, with about 1% of the general population being affected, only 10–20% of people appear to be aware of their condition and follow a strictly gluten-free diet [209]. Mild forms of Celiac's disease, however, have the potential to worsen with age, thus, management through a gluten-free diet is necessary to decrease severe complications, such as osteoporotic fractures and intestinal cancers [209].

Interestingly, there are also other clinical presentations that do not fully correspond with Celiac's disease but rather consist of new clinical syndromes, typically termed non-celiac gluten and/or non-celiac wheat sensitivity. Though controversial and under-studied, it is generally accepted that these syndromes exist, but in the absence of gluten-ingested, celiac-specific antibodies [210] or wheat allergies [192]. What ultimately triggers these syndromes is unclear, as it may not need to be gluten but could include other components of wheat, such as the low fermentable, poorly absorbed, short-chain carbohydrates (FODMAPs) [211], other proteins [212], or insecticides such as glyphosate [29,30].

Regardless of exact mechanism, variability in clinical symptoms from grain or wheat ingestion pose new opportunities for nutrition professionals, considering both, the recent changes in clinical presentations and the modernization of many plants, including wheat [192]. While challenging, this should provide new avenues for dietary management of those who prefer a gluten-free diet for performance enhancement or health promotion, in the absence of Celiac's disease, to trial various approaches [192,207,213,214], as opposed to eating a strictly gluten-free diet. A gluten-free diet per se, with a high amount of processed gluten-free foods, may not meet nutritional recommendations, and a gluten-free, vegan diet could pose serious negative health and performance effects (e.g., B-vitamin deficiency). It has also been suggested that individuals should choose grains and their processing wisely, as this may reduce the risk of developing Celiac's disease in those who may have hereditary risk [202]. As ancient and heritage grain production is sweeping through the United States as a long-awaited player in the local food movement, the grain chain, from farmer to baker to table, is filled with food literacy opportunities for athletes such as making bread together. After all, bread has been a staple around the world with thousands of traditional uses. Bread is also one of the primary carbohydrate choices for athletes in training and competition, and carbohydrate is the major source of calories for most humans [113], including athletes [215].

While ancient and heritage grains are making their way back to the grocery stores, their production remains relatively small. However, these grains are known to be more drought tolerant [198] and using grains in crop rotation or as cover crop can meaningfully contribute to farm diversification and sustainable agriculture [78]. Grain production may soon take a turn for the better and become more sustainable as scientists at the Land Institute in Salina, Kansas [216] will likely announce that perennial grains (long roots capture carbon, enhance soil quality, and help reduce erosion) may replace modern wheat, not only on the field but also in people's bread baskets.

3.5. Food Literacy and Food Citizenship in Sports and Exercise

There is much to relearn when it comes to food. Perhaps we have moved away too far from field, farm, and the kitchen to know where food comes from and when it is in season. We also have lost important life skills such as cooking. We have to relearn and teach these simple skills to rebuild the knowledge needed to establish a healthy relationship with food. This brings us to the topic of food literacy. Recently, Vidgen and Gallegos, 2014 defined food literacy as the following:

"Food literacy is the scaffolding that empowers individuals, households, communities or nations to protect diet quality through change and strengthen dietary resilience over time. It is composed of a collection of inter-related knowledge, skills and behaviors required to plan, manage, select, prepare and eat food to meet needs and determine intake. This can simply be translated as the tools needed for a healthy lifelong relationship with food" [217] (p. 54).

Academic programs that promote food literacy, through curricula that meet joint goals of health promotion and sustainable development, especially in the health professions, may allow for transformative experiences [74]. Such food literacy discourse has the ability to diffuse, with outcomes that promote food citizenship in young people, and therefore, future generations [218,219]. Food citizenship is the practice of engaging in food-related behaviors (defined narrowly and broadly) that support, rather than threaten, the democratic, socially and economically just, and environmentally sustainable food systems [220] (p. 271).

Athletes and their support staff should be introduced to the link between daily food choices, health, and sustainability. It is most likely the sports dietitian who will bring this topic to the table, and the best and least confrontational approach, may be through a sustainably sourced meal cooked together such as a "Team Dinner" or a fun food literacy event with multiple stations, competitive team work, and food-related prizes. Shopping at local food outlets, including the farmer's market, and cooking together might be other options to open the dialogue pertaining to sustainable quantity and quality of food, as discussed above. Eating practices and fueling strategies are a performance-determining factor; however, becoming a food citizen [221] with knowledge and skill to navigate through an ever more complex food web opens the narrow sports-performance focus of a young athlete and introduces areas such as environmental conservation. Thus, sport nutrition education should begin to integrate sustainable food topics and promote food citizenship and food literacy by an enabling, participatory approach when the timing is right and where opportunities arise.

3.5.1. Athletes to Farm

While sport nutrition is a broad field and athlete performance and health issues take precedent over sustainability efforts, the sports dietitian will need to find a good balance that allows for sustainability integration, without feeling constrained but rather enabled in promoting awareness, building knowledge, and enhancing skills around food, ultimately improving dietary habits of young people. Thus, going to a local farm and/or market to buy food is only the first stop in this refreshed sport nutrition curriculum. While athletes often crave for the latest in exotic products from far away (e.g., Acai berries), eating some of the unfamiliar and wild foods grown close to home, may not only be more nutritious, but will also come with a plethora of learning opportunities. To allow athletes to make a connection with their home environment through the farmers who grow their food, training plans may need to be flexible to allow for Community Supported Agriculture (CSA) share pick up, a farmer's

market visit, or a farm-field training day, as this may offer invaluable experiential nutrition education. The opportunity for athletes to experience "local life" is short but is increasingly meaningful, as sport teams and elite athletes are in the spotlight at home. Engaging with the local community may bring personal and team-related benefits for farm-fresh food support that has the potential to strengthen athletes' community involvement and build a sense of place. Finally, investing in the community, through food procurement from local farms, may also set the precedent for a supportive environment should athletes get injured or to facilitate the transition from athletic to normalized life after the career is concluded.

Eating locally grown and raised food has many benefits, but it may not automatically be more environmentally sustainable. Nevertheless, the local food movement might ignite people's desire for better taste, connection to place and to the people in their community. Local food seems to attract people also because of its economic benefit to the community, and there is a general sense, despite the fact that local food often costs more, that it is more affordable [222]. Regardless, those having worked and experienced the local food movement cannot let it go, and while the urgency to become more food secure in this changing world calls for revolutionary action through more sustainable food production [11], engaging in local food mobilizes people on a deeply emotional level, often difficult to express for those who are in it [223], but likely the reason why people may identify it as a realistic way to change eating behavior [55]. Recent research also shows that those buying direct from the farmer think and act around food very differently, compared to those going to a chain grocery store to procure their food [221]. Thus, the local food system is engaging inter-personally and economically within a community and it also teaches about food, the seasons, biodiversity, flavors, nutrition, cooking, culture and tradition.

While not always the most sustainable, the local food system may be a vehicle that could direct people to healthier and more mindful eating. A recent study illustrates how the awareness of dietary choices and eating can meet joint goals of individual health, environmental sustainability, and food security [224]. Thus, the many facets of a local food system can act as living learning laboratory to practice mindfulness training, even in athletes and their teams, as they cultivate both eating for sport and eating for planet Earth.

Local food systems are defined as "collaborative effort in a particular place to build more locally based, self-reliant food systems and economics—one in which sustainable food production, processing, distribution and consumption is integrated to enhance the economic, environmental and social health of a particular place." [225] (p. 100).

If athletes receive food money, a resource factsheet with local food procurement options could begin the collaboration with local business. A factsheet could also promote best choices when shopping at grocery stores, how to identify what's locally produced, what's seasonal, which labels to observe (e.g., USDA Organic; Buy Local; Marine Stewardship Council, MSC or Aquaculture Stewardship Council, ASC; GMO Free Project; Humanely Raised, American Grassfed, Direct or Fair Trade), the list of the dirty dozen [226], and how to order in bulk online, including heritage/ancient grains. Identifying farm-team partnerships requires farm visits and direct communication with the farmers [227]. In addition, providing some community service at the farm with 1 or 2 workouts held at the farm per year, supporting planting, weeding, or harvesting, will facilitate access to local, farm-fresh food because a connection is built much to the delight of the small-scale farmer who feels supported by the local sports team. Teams may also obtain group discounts if ordering in bulk through local buying clubs, food hubs, or food cooperatives. With CSA shares, there is great flexibility should shares get temporarily suspended when athletes travel. It is also possible to obtain surplus food and getting parents involved to preserve this food for later. Preservation, including fermentation, will not only support nutrition programming, but could be applied at times of increased team stress when athletes' immune function is more susceptible to illness. Locally, seasonally, and organically grown produce is more nutritious [175,179,180,188,228] and fermentation (e.g., pre- and probiotics) may add

immune [229,230] support in times when athletes need it. Check with local University Extension offices for safe guidelines on canning and fermentation.

3.5.2. Taste Education and Cooking

Taste education with athletes can be integrated at any time, combined with a general team talk (locally grown fruit, vegetables, or grains as tasters), fueling or recovery workshops (integrating seasonal fruit, yogurt, and honey), or even during a travel nutrition talk (cultural food tasting of the travel destination). Nutrition should no longer be taught without hands-on learning from farm to kitchen. Written or visual materials (e.g., posters) or recipes that integrate local producers, topics of food citizenship (e.g., farmer's market shopping), or health benefits of diet diversity (e.g., biodiversity of greens) will keep building awareness and return home economics to young people's lives. Edible nutrition education is not only fun, inspiring, and tasty but it also teaches young people important skills and it builds a lifelong healthy relationship with food—and that is food literacy [217]. In addition, working with a farm-to-training table curriculum in sports also provides an opportunity to highlight local producers, dairies, farmers, or bakers, the history of the place, and this brings meaning and relational values [231]. If time is tight, University nutrition programs may partner to support a revisited curriculum that integrates agriculture and culinary training. One such example is the Flying Carrot Food Literacy Truck [232]. This program has been led by graduate students in sport nutrition at UCCS for the past 5 years [233]. After initial inception, several food-related courses, internships, and service learning experiences within the Southern Colorado regional food system, including a campus farm with its farm-to-table café, Food Next Door [134], and local food literacy farmhouse, are now serving a vigorous on-farm and in-kitchen curriculum for undergraduate and graduate students at UCCS, some of whom are in sport nutrition.

3.5.3. Budgets, Planning, and Food Waste

Food literacy should also integrate the full circle of engaged eaters' choices, including the discussion of food waste. Athletes and their families may tap into rescued food programs if budgets are tight. Most cities today have food rescue programs and some cities and programs, such as the one known as P.O.W.W.O.W. by the Borderland Foodbank in Southern Arizona [234], have made it possible to access fresh food, at affordable price, that otherwise would go to landfill. Food waste at the consumer level originates especially due to consumers' aesthetic preferences and arbitrary sell-by dates [36] as well as simply by purchasing, cooking, preparing and serving too much [39]. Teaching athletes to purchase what they can eat, cook what they purchase and promoting safe preservation and freezing techniques, are all part of food literacy training. The sports dietitian can help with weekly planning, providing input with shopping and cooking, so that athletes learn when, what, and how much to cook and to plan their dietary strategies, as much as the coach plans their training schedule. Cooking and planning ahead has been identified as a critical strategy to reducing food waste on the consumer level [39]. When traveling, a little bit of research ahead of time will pay off. Food cooperatives often have restaurants and there are many "Pay-What-You-Can" non-profit community restaurants in the US that serve local and organic food, often rescued from what would have otherwise been wasted, and sold at very low price (or what the team can pay [235]). These types of food outlets, including food "waste" supermarkets, are becoming more available everywhere. Obviously, each such stop will add to nutrition education and athletes learn they can eat this way everywhere they go. For good restaurant, market, and café guides that serve local, sustainable, and organic food, see Slow Food USA or Edible Communities [236,237].

3.6. Timing of Sustainability Integration in Exercise and Sports

In this paper, we addressed the environmental impact of food choices, easy changes that can be made (e.g., eating less meat), and paying attention to the food value chain to obtain high quality food with zero waste strategies. We have also integrated the local food system as a great entry way

to connect sustainability and health, leveraging co-benefits for both planet and people. Posing the question on when to integrate sustainability principles in sports nutrition may sound as if athletes and sport teams have a special status concerning the food of the future. The answer is, nobody does, and shifting to a low-carbon consumer culture is a necessity rather than a choice. However, there needs to be careful consideration when to launch or what to initiate within the economic boundaries of grass-root sports, where parents are the coaches and kids are running from A to Z with plastic wrappers in their hands, squeezing out their pre-game meal. Likewise, timing considerations on the elite level must involve everyone because the budgets will have to, at least in part, account for increased food costs, cooking and team dinners, and time to pick up fresh food at farmers' markets, farm stands or neighborhood stores.

The best timing to plan any new programs within the world of sports is usually as the season is coming to an end and early before the start of the next training cycle. This is especially true should extra resources be needed to support the program. Farm CSA shares cost between $500–$600 for 6–8 months or about $20–$30 weekly, with each share providing food for about 4 people. Team talks with edible tasters will either require planning and connecting with local producers for samples or more expensive transactions at the store or market. Thus, the more time is invested to form farm-to-sports partnerships, the better and more economical the outcome.

All athletes spend time training at home. This is the best time to teach shopping and cooking. Depending on the season, it is also the best time to introduce local food with farm and market visits. Even though athletes are still on the go every day while in training, there is the potential for community connections through the local food system. Thus, providing a platform for this to occur may create a new sense of purpose, external to the identity of being an athlete. Participating in the community may balance the lives of the elite and new friendships may arise with those who work the land, which may create awareness of earth stewardship and food citizenship.

Once a program launches it is difficult to hold it back and it will evolve on its own. This is especially true for the local food movement. It needs ignition, but once the web is being explored and experiences are made, there is no going back. It is a paradigm shift. It's a local food revolution [223].

Should sustainability be a topic while traveling? The answer is yes, because in many countries, sustainable food systems are still the norm. Thus, traveling to European and Eastern European countries is often an eye-opener. Taking athletes into the grocery stores or through a local market is food literacy away from home, and sports dietitians also increase their knowledge and skills when exploring foods abroad. While most travels abroad are hectic with little time, surprisingly, the Olympics may be the perfect place for food literacy. The local volunteers are a great resource for information and they provide access to local markets to purchase fresh food. Thus, even when traveling, there are multiple opportunities to broaden food experiences and teach important cultural food differences.

Finally, introducing sustainability in sport nutrition may also be timely for those who are injured. These athletes may have more time and interest to learn about whole, nutritious food and cooking that could enhance healing. In addition, introducing athletes to other areas outside of sport, such as agriculture or cooking, may distract the overly occupied mind, and help maintain a positive attitude during the recovery and return-to-play period.

Taken together, while the timing of sustainability integration must be carefully considered to bring change to nutrition programming for athletes, small steps can fit everywhere and they bring with them deliciousness, beauty, and inspiration to participate in the food chain from farm to kitchen and table. There should be no doubt that this is the future of how nutrition should be taught, also in sports.

3.7. Integration of Sustainability Practices as Collective Commitment in Sports

3.7.1. Team Sustainability

Integrating sustainability in the sport nutrition program benefits first the athlete. However, coaches and other members of the sport science team, including athletic trainer, sports medicine doctor, and psychologist all benefit. Because of the performance enhancing team

approach and multi-disciplinary strategies, sustainability and food will also open the dialogue of sustainable practices in general. This may mean that the team develops a vision or even a policy for sustainable development, especially considering training venues at home, where more influence is possible. Starting with food and drink, this may mean the team implements a recycling, re-using, and composting strategy. It may mean the team bans bottled water and throw-away, take-out containers. And it may mean preferred vendors for training tables or team meetings come from local businesses, using sustainably and locally sourced food. Catering may be enhanced through the-less-but-better meat initiative in combination with highly nutritious grains and beans, seasonal vegetables and fresh fruit. Team commitments may also include coach and support staff's eating practices that are coherent with the underlying philosophy of eating for performance and health. Finally, taking on a team vision for a sustainable future may also inspire parents and families and this could be supported by social media and website resources. A great example of how sustainability can be part of every sporting venue is the Green Sports Alliance [238].

3.7.2. Institutional Sustainability

Whether it is at a high school, university, or national/regional/local sport center level, integrating sustainable food procurement into food service starts to open many opportunities. It allows for a new seasonal menu. Reducing meat through the protein flip and boosting vegetarian offerings, sparks creativity in chefs and curiosity in athletes. Sourcing locally brings in the story of the farmer, unknown diet diversity, and awareness related to the link between fresh food and health on an individual, community, and environmental level. If institutions have gardens or farms, there is potential to integrate edible education linked to the menu served, in addition to the invaluable seed--to-plate menu. However, change is always more challenging than we think. Thus, to initiate a new menu, it is crucial that athletes and coaches understand the rationale behind the change. If resistance develops, athletes could be integrated in various educational activities that incorporate their own food preferences, cooking competitions, or recipe contests. It is helpful for athletes to see protein numbers of a meal and over a day to reduce fear of not getting enough. From a health perspective, there are many opportunities when food service commits to a more sustainable menu, with procurement gradually shifting to seasonal, organic, local, pasture-fed, free-range, and sustainably produced, fished or farmed food.

3.7.3. Event Sustainability

Integrating sustainable food into sporting events is being done on many levels. Some examples include London 2012 [239] and Rio 2016 [240]. Both local organizing committees published their sustainable food visions and made procurement with sustainable agricultural standards a priority. Especially the Rio Games were impressive as to the portrayed commitment to environmental consciousness through sustainable sourcing, improving supply chains, managing packaging, and reducing waste. As previously discussed, Brazil is one of the few countries whose governmental guidelines have embraced sustainability [56]. Whether visions and guidelines are ultimately implemented at the international events is difficult to tell, as there has been no labeling that details sustainable sourcing. This has previously been noted and published by Pelly et al., 2014 based on a survey conducted by sports dietitians, representing various countries at the 2012 London Olympic Games [241]. While the international sport nutrition organization, Professionals in Nutrition for Exercise and Sport (PINES) [242] reviews the menus for each Olympic cycle, an on-site implementation phase could help improve both menu and labeling, with inclusion of sustainable sourcing. In addition, the athlete dining hall and the Olympic village present an enormous challenge to sustain environmental commitments, considering food waste, bottled beverages, and to-go meals. In the future, food service at the Olympic Games should promote sustainability more visibly, highlighting a country's food culture and offering athletes experiential learning opportunities that showcase regional food traditions, seasonality, world heritage, and the story of farmers. Tokyo 2020 would be an excellent host city

to bring change to the athlete dining hall with greater transparency for sourcing, local food literacy, and hands-on learning (e.g., how to make tofu or soba). The Olympics are long and many athletes have downtime. Why not learn something about the host country's food culture, sustainability efforts, seasonality of food, and how traditional foods are produced? While currently implemented at the Youth Olympic Games, integrating the host country's food traditions could augment the cultural experience of athletes visiting the Olympic village dining hall.

4. Conclusions

Environmental impact of food production is high, especially when considering the GhG emissions, land, and water use of animal agriculture. Many governmental organizations are beginning to integrate sustainability into their dietary guidelines and are calling on consumers to eat less animal and more plant-based foods. Integrating health and sustainability creates co-benefits, as for the most part, sustainable eating also means healthful eating. Nutrition recommendations, for active and athletic individuals should also begin to integrate sustainability. Using innovative approaches, including experiential learning from farm to table, renews the relationship of food by rediscovering the broad meaning of food, building knowledge and skills in the kitchen, and sharing food around the table. Initiating sustainable practices in sport, including sustainable food procurement, opens many opportunities for athletes and their entourage to engage in local and regional food systems, and by curbing the appetite for meat, individuals, teams, institutions and organizers begin to contribute to a reduction in global warming from the food sector.

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6

Pre-Sleep Protein Ingestion to Improve the Skeletal Muscle Adaptive Response to Exercise Training

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Abstract: Protein ingestion following resistance-type exercise stimulates muscle protein synthesis rates, and enhances the skeletal muscle adaptive response to prolonged resistance-type exercise training. As the adaptive response to a single bout of resistance exercise extends well beyond the first couple of hours of post-exercise recovery, recent studies have begun to investigate the impact of the timing and distribution of protein ingestion during more prolonged recovery periods. Recent work has shown that overnight muscle protein synthesis rates are restricted by the level of amino acid availability. Protein ingested prior to sleep is effectively digested and absorbed, and thereby stimulates muscle protein synthesis rates during overnight recovery. When applied during a prolonged period of resistance-type exercise training, protein supplementation prior to sleep can further augment gains in muscle mass and strength. Recent studies investigating the impact of pre-sleep protein ingestion suggest that at least 40 g of protein is required to display a robust increase in muscle protein synthesis rates throughout overnight sleep. Furthermore, prior exercise allows more of the pre-sleep protein-derived amino acids to be utilized for de novo muscle protein synthesis during sleep. In short, pre-sleep protein ingestion represents an effective dietary strategy to improve overnight muscle protein synthesis, thereby improving the skeletal muscle adaptive response to exercise training.

Keywords: sleep; recovery; exercise; hypertrophy; casein

1. Introduction

A single session of exercise stimulates muscle protein synthesis rates, and to a lesser extent, muscle protein breakdown rates [1,2]. However, the muscle protein net balance will remain negative in the absence of food intake [2]. Protein ingestion stimulates muscle protein synthesis and inhibits muscle protein breakdown rates, resulting in net muscle protein accretion during the acute stages of post-exercise recovery [3]. Therefore, post-exercise protein ingestion is widely applied as a strategy to augment post-exercise muscle protein synthesis rates and, as such, to facilitate the skeletal muscle adaptive response to exercise training. Various factors have been identified which can modulate the post-exercise muscle protein synthetic response to exercise including the amount [4,5], type [6,7], timing [8], and distribution [9] of protein ingestion.

Only few studies have investigated the dose-response relationship between protein ingestion and post-exercise muscle protein synthesis rates in young [4,5] and older adults [10–12]. Ingestion of 20 g egg or whey protein has been shown sufficient to maximize muscle protein synthesis rates during recovery from lower-body resistance-type exercise in young males [4,5]. More recent evidence indicates that this dose-response relationship may depend on the amount of muscle tissue that was recruited during exercise, with the ingestion of 40 g protein further increasing muscle protein synthesis rates during recovery from whole-body resistance-type exercise [13].

A large variety of dietary protein sources have been shown to stimulate post-exercise muscle protein synthesis rates, including egg protein [4], whey and casein protein [14], milk and beef protein [15], and soy protein [6]. However, dietary protein sources can differ in their capacity to stimulate muscle protein synthesis rates, which appears to be largely dependent on differences in protein digestion and absorption kinetics [14,16] and amino acid composition [6,17], with the leucine content being of particular relevance [18,19].

Besides the amount and type of ingested protein, the timing and distribution of protein ingestion throughout the day can modulate post-exercise muscle protein synthesis rates. An even distribution of total protein intake over the three main meals stimulates 24 h muscle protein synthesis rates more effectively than an unbalanced distribution in which the majority (>60%) of total daily protein intake is consumed at the evening meal [20]. During 12 h of post-exercise recovery, an intermediate pattern of protein ingestion (20 g every 3 h) seems to increase muscle protein synthesis rates to a greater extent than the same amount of protein provided in less frequent but larger amounts (40 g every 6 h), or in more frequent, smaller amounts (10 g every 6 h) [9]. Therefore, an effective pattern of daily protein intake distribution to support muscle protein synthesis is to provide at least 20 g of protein with each main meal with no more than 4–5 h between meals.

As overnight sleep is typically the longest post-absorptive period during the day, we have recently introduced the concept of protein ingestion prior to sleep as a means to augment post-exercise overnight muscle protein synthesis. The aim of this review is to discuss the current state of evidence regarding the efficacy of pre-sleep protein ingestion to stimulate overnight muscle reconditioning.

2. Overnight Protein Metabolism

In general, most studies assess the effects of food intake on the muscle protein synthetic response to exercise performed in an overnight fasted state. Such post-absorptive conditions differ from normal everyday practice in which recreational sports activities are often performed in the late afternoon or evening after a full day of habitual physical activity and food intake. Therefore, we evaluated the impact of exercise performed in a fed state in the evening and the efficacy of protein ingestion immediately after exercise on muscle protein synthesis during prolonged overnight recovery [21]. The ingestion of 20–25 g of protein during exercise increased muscle protein synthesis rates during exercise, but we observed no increase in muscle protein synthesis rates during the prolonged overnight recovery period. Muscle protein synthesis rates during overnight sleep were unexpectedly low, with values being even lower than those typically observed in the in the morning following an overnight fast. Thus, a day of habitual food intake and the ingestion of 20–25 g of protein during and/or immediately after an exercise bout performed in the evening does not suffice to augment overnight muscle protein reconditioning.

3. Does the Gut Function at Night?

As overnight muscle protein synthesis rates are surprisingly low [21], we questioned whether they are limited by overnight plasma amino acid availability. Therefore, we hypothesized that protein provision during sleep increases overnight plasma amino acid availability and stimulates overnight muscle protein synthesis rates. As human intestinal motility follows a circadian rhythm with reduced activity during the night [22], we first assessed whether dietary protein provision during sleep leads to proper dietary protein digestion and amino acid absorption. In a proof-of-principle study, we first administrated specifically produced intrinsically L-[1-^{13}C]-phenylalanine-labeled casein protein via a nasogastric tube while subjects were asleep and assessed the subsequent protein digestion and absorption kinetics [23]. We observed that administration of 40 g casein via a nasogastric tube during overnight sleep is followed by proper dietary protein digestion and absorption kinetics, thereby increasing overnight plasma amino acid availability and increasing muscle protein synthesis rates. Clearly, these data demonstrated that the gut functions properly at night and that protein provided during sleep strongly increases overnight muscle protein synthesis rates.

4. Pre-Sleep Protein Feeding as a Strategy to Increase Overnight Muscle Protein Synthesis

Our observation that protein administered during sleep is effectively digested and absorbed provided proof-of-principle that the gut functions properly during sleep [23]. However, nasogastric tube feeding does not represent a feasible feeding strategy for athletes. Therefore, our next step was to assess if protein ingestion prior to sleep would represent an effective dietary strategy to increase muscle protein synthesis rates during overnight post-exercise recovery [24]. Therefore, we studied recreational athletes during overnight recovery from a single bout of resistance-type exercise performed in the evening after a full day of dietary standardization. Immediately after exercise, all athletes ingested a recovery drink containing 20 g protein to maximize muscle protein synthesis rates during the acute stages of post-exercise recovery [4,24]. As explained above, this prescribed recovery strategy does not suffice to maintain elevated muscle protein synthesis rates during more prolonged overnight sleep [21]. Therefore, we provided subjects with either 40 g casein protein or a placebo drink immediately prior to sleep. In line with intragastric protein administration during sleep [23], the bolus of protein ingested prior to sleep was properly digested and absorbed throughout overnight sleep. The greater plasma amino acid availability following pre-sleep protein ingestion improved the overnight whole-body protein balance, allowing the net protein balance to become positive. In line, muscle protein synthesis rates were approximately 22% higher during overnight recovery when protein was ingested prior to sleep when compared to the placebo treatment. From these data we concluded that pre-sleep protein ingestion represents an effective dietary strategy to further augment the skeletal muscle adaptive response to resistance-type exercise training (Figure 1).

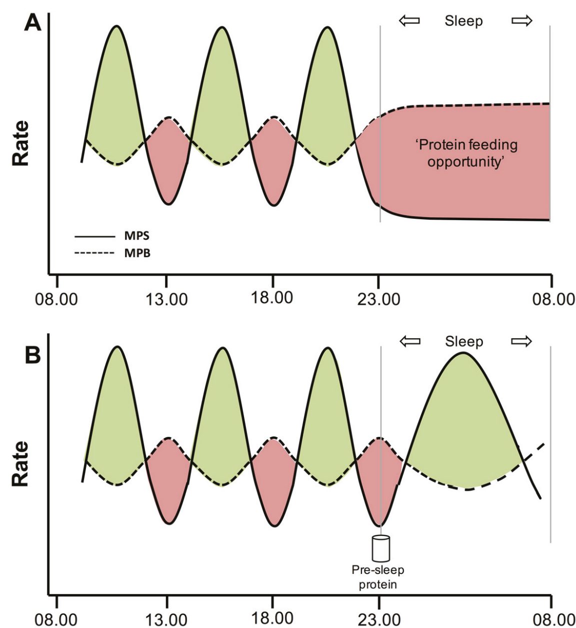

Figure 1. Schematic representation of the process of muscle protein synthesis (MPS) and muscle protein breakdown (MPB) throughout the day. Protein ingestion stimulates MPS rates and allows for net muscle protein accretion (green areas). During post-absorptive conditions, MPB rates exceed MPS rates, resulting in a net loss of muscle protein (red areas). Overnight sleep is the longest post-absorptive period of the day (**A**). Pre-sleep protein ingestion stimulates overnight muscle protein synthesis rates (**B**), thereby improving muscle reconditioning during overnight sleep.

To test this hypothesis, we assessed the impact of pre-sleep protein feeding to facilitate the skeletal muscle adaptive response to prolonged resistance-type exercise training [25]. Specifically, we selected healthy young men to participate in a 12-week resistance-type exercise training program (three exercise sessions per week) during which they ingested either 27.5 g of protein prior to sleep, or a non-caloric placebo. Muscle mass and strength increased to a greater extent in the group that ingested protein prior to sleep. These results indicate that protein supplementation prior to sleep represents an effective dietary strategy to augment the gains in muscle mass and strength during resistance-type exercise training. It remains to be established what dose and type of pre-sleep protein should be used to further optimize overnight muscle protein synthesis rates and, as such, can support greater gains in muscle mass and strength.

It should be noted that the ingestion of the pre-sleep protein supplement in both our acute and long-term studies was compared with a non-protein placebo, and not compared with protein supplementation provided at other time points. Therefore, we can only speculate on the surplus benefits of pre-sleep protein provision when compared to other time points. It can be speculated that the greater gains in muscle mass and strength are, at least partly, attributed to the pre-sleep timing of the protein supplement, as the vast majority of studies in which protein has been supplemented immediately before and/or after exercise do not show an increase in muscle mass gains when compared to a placebo [26]. However, it has been suggested that protein supplementation increases muscle mass gains mainly as a function of increased total protein intake, rather than the specific timing of a protein supplement [27,28]. As a meta-analysis was required to demonstrate that additional protein intake augments training-induced muscle hypertrophy [26], it seems unlikely that a possible positive effect of protein timing (i.e., protein supplementation at a time point compared to protein supplementation at different time point) on muscle mass gains can be detected in a longitudinal study. While it is currently unclear whether pre-sleep protein ingestion is superior to protein ingestion at a different time point, we propose that a more relevant question is whether pre-sleep protein ingestion is additive to protein intake earlier in the day. We suggest that athletes should aim to ingest sufficient protein intake at every meal to maximize muscle protein synthesis until the next meal. We have recently shown that the ingestion of large amounts of protein in the early post-exercise recovery phase does not compromise the muscle protein synthetic response to protein ingestion at a later stage [29]. This suggests that every meal moment represents a unique opportunity to stimulate muscle protein synthesis and that the muscle protein synthetic response to each meal may be additive. In addition, we have recently shown that athletes typically consume well above 1.2 g protein/kg/day, with the majority of protein consumed during the three main meals, and only a small amount of protein eaten as an evening snack (~7 g) [30]. As such, additional pre-sleep protein ingestion represents a practical strategy to increase the total daily protein intake, add another meal moment, and increase the overnight muscle protein synthesis rates; this effect is likely additive to muscle protein synthesis rates observed throughout the day.

5. Pre-Sleep Protein Feeding Characteristics

While we have identified the overnight sleeping period as a new window of opportunity to augment post-exercise training adaptations, it remains to be established how we can maximize the impact of pre-sleep protein feeding on overnight muscle protein synthesis rates. Previously we have shown that the ingestion of 40 g protein prior to sleep stimulates overnight muscle protein rates [24], which is considerably more than the 20 g of protein that is supposed to maximize muscle protein synthesis rates during the first few hours of post-exercise recovery [4,5]. Therefore, we questioned if a more moderate amount of protein would suffice to augment overnight muscle protein synthesis rates. To address this issue, we performed a follow-up study similar in design to our previous pre-sleep protein work, with the main difference that we provided 30 g of highly enriched intrinsically labeled protein prior to sleep, with or without an additional 2 g of free leucine. In contrast to our previous findings with 40 g protein, the ingestion of 30 g protein prior to sleep did not significantly increase

overnight muscle protein synthesis rates (preliminary observations). This suggests that a pre-sleep protein dose-response relationship exists, which differs from the immediate post-exercise recovery period during which the ingestion of merely 20 g protein seems to maximize post-exercise muscle protein synthesis rates in young adults.

The ingestion of highly enriched, intrinsically L-[1-^{13}C]-phenylalanine-labeled protein allowed us to also directly assess the metabolic fate of the pre-sleep dietary protein-derived amino acids. Pre-sleep protein-derived L-[1-^{13}C]-phenylalanine was incorporated in de novo muscle protein as evidenced by the increase in muscle protein–bound L-[1-^{13}C]-phenylalanine following overnight recovery, demonstrating that the pre-sleep protein provided amino acids as precursors for de novo myofibrillar protein accretion during overnight sleep. This provides mechanistic evidence to support our observation that the ingestion of 30 g protein prior to sleep augments muscle mass during three months of resistance-type exercise training [25]. However, our data suggest that at least 40 g of pre-sleep protein is required to induce a more substantial, detectable increase in muscle protein synthesis rates when assessed acutely over a 7.5 h overnight period.

As we anticipated that 30 g of pre-sleep protein might not be sufficient to adequately increase overnight muscle protein synthesis rates, we included a third treatment in which 2 g crystalline leucine was added to the 30 g bolus of protein. The addition of supplemental free leucine to a suboptimal amount of protein has been shown to enhance post-exercise muscle protein synthesis rates [18,19,31,32]. Despite these previous observations, co-ingesting free leucine with 30 g of casein prior to sleep did not augment the overnight muscle protein synthetic response. Given the extended duration of overnight sleep compared to a typical postprandial period (8 vs. 4–5 h), it is tempting to speculate that larger amounts of protein ($\geq$40 g) are required to maximize muscle protein synthesis rates during overnight sleep.

6. Prior Exercise

It has been well established that the muscle protein synthetic response to protein ingestion is enhanced following exercise when exercise is performed in the morning following an overnight fast [12,33]. Recently, we evaluated the effect of resistance-type exercise performed in the evening on the muscle protein synthetic response to pre-sleep protein ingestion [34]. Postprandial overnight muscle protein synthesis rates were higher when exercise had been performed earlier that evening and more of the ingested protein-derived amino acids were directed towards de novo myofibrillar protein synthesis during overnight sleep. Therefore, protein ingestion prior to sleep represents an effective strategy to enhance overnight muscle reconditioning and is likely of even more relevance on exercise training days. In line, we have shown that physical activity performed in the evening increases the overnight muscle protein synthetic response to pre-sleep protein ingestion in older adults [35]. Clearly, combing pre-sleep protein ingestion with resistance-type exercise represents a more effective strategy to further enhance overnight skeletal muscle protein synthesis rates and increases the efficiency by which dietary protein is used for muscle protein accretion (Figure 2).

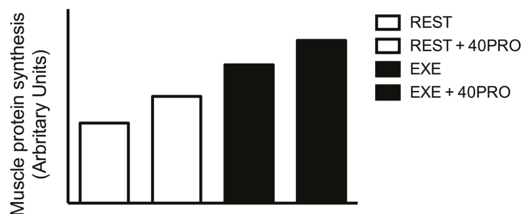

Figure 2. Conceptual framework of the overnight muscle protein synthetic response to 40 g of pre-sleep protein feeding at rest or following prior exercise.

7. Type of Pre-Sleep Protein

As protein sources differ in their capacity to stimulate muscle protein synthesis, the type of protein ingested prior to sleep may modulate the overnight muscle protein synthetic response. So far, all studies assessing the efficacy of pre-sleep protein ingestion on exercise reconditioning have provided casein protein. Casein is a more slowly digestible protein source, allowing a more moderate but prolonged rise in plasma amino acid concentrations [17]. Given the extended nature of overnight sleep, it could be speculated that such a more sustained postprandial aminoacidemia during overnight sleep is preferred as it will provide precursors to support muscle protein synthesis rates throughout the entire night. In contrast, whey protein is a more rapidly digestible protein, resulting in a pronounced but transient rise in plasma amino acid concentrations [17]. Ingestion of a single bolus of whey protein has been shown to stimulate muscle protein synthesis rates to a greater degree than casein protein when assessed over periods up to 6 h [6,17,36]. This has been attributed to the more rapid protein digestion and amino acid absorption kinetics as well as the higher leucine content in whey versus casein protein, resulting in a more rapid rise in postprandial plasma leucine concentrations [37]. It remains to be established if whey is superior to casein protein when ingested prior to sleep and muscle protein synthesis rates are assessed over a more prolonged overnight period of 7.5 h. The plasma levels of leucine do not seem to be the only factor in this regard, as we recently did not observe any differences in overnight muscle protein synthesis rates following the ingestion of 30 g casein with or without 2 g crystalline leucine (preliminary observations). Snijders et al. [25] provided a casein protein supplement that consisted of 50% micellar casein and 50% casein hydrolysate. When casein protein is hydrolyzed, its digestion and absorption properties resemble a more rapid digestible protein [38]. Therefore, pre-sleep ingestion of a mixture of a slow and more rapidly digestible protein source appears to be effective to augment muscle mass and strength gains during a prolonged resistance-type exercise program. We speculate that a variety of high-quality animal-based protein sources can augment overnight muscle protein synthesis rates when provided in sufficient amounts ($\geq$40 g; Table 1), with relatively minor differences in efficacy between sources.

Table 1. Quantity of protein sources to provide 40 g pre-sleep protein.

Food Item	Quantity
Cooked eggs	7 eggs
Low fat milk	5 cups (1025 mL)
Low fat yogurt	5 cups (1176 mL)
Chicken breast	2 breasts (176 g)
Steak	2 steaks (168 g)
Protein concentrate in water	3 scoops (60 g)
Protein concentrate in low-fat milk	2 scoops in 300 mL

8. Applications

Overnight sleep has emerged as a novel window of opportunity to modulate muscle protein metabolism. Pre-sleep protein ingestion represents an effective dietary strategy to stimulate both the acute and long-term skeletal muscle adaptive response to resistance-type exercise training [24,25]. There are numerous other potential applications of protein ingestion prior to sleep. Protein ingestion prior to sleep may also enhance exercise training adaptations to other exercise modalities. However, research on the impact of protein supplementation on other modes of exercise such as concurrent training [39] or endurance-type exercise training [40] is surprisingly scarce. While protein ingested immediately after endurance-type exercise does not appear to further augment mitochondrial protein synthesis rates [40], amino acid administration at rest stimulates mitochondrial protein synthesis rates [41]. It remains to be established if pre-sleep protein can augment the adaptive response to endurance-type exercise training with greater increases in skeletal muscle oxidative capacity, vascular density and/or endurance performance capacity.

Protein administration during sleep has been shown to stimulate overnight muscle protein synthesis rates in older adults [23]. Consequently, pre-sleep protein feeding may also represent an effective interventional strategy to support muscle mass maintenance in the older population or possibly even in patients in more clinically compromised conditions characterized by accelerated muscle loss such as acute sickness, systematic inflammation, and muscle disuse [42,43].

9. Conclusions

Muscle protein synthesis rates are particularly low during sleep, even when 20 g protein is ingested immediately after exercise performed in the evening. Protein ingested immediately prior to sleep is effectively digested and absorbed, thereby increasing amino acid availability during overnight sleep. Greater amino acid availability during sleep stimulates muscle protein synthesis rates and improves whole-body protein net balance during overnight recovery. At least 40 g of dietary protein should be ingested prior to sleep to elicit a robust stimulation of muscle protein synthesis rates throughout the night. Resistance-type exercise performed during the day augments the overnight muscle protein synthetic response to pre-sleep protein ingestion and allows more of the protein-derived amino acids to be used as precursors for de novo muscle protein synthesis. When applied during prolonged resistance-type exercise, pre-sleep protein supplementation can be used effectively to further increase gains in muscle mass and strength.

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Effects of Carbohydrate and Glutamine Supplementation on Oral Mucosa Immunity after Strenuous Exercise at High Altitude: A Double-Blind Randomized Trial

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Abstract: This study analyzed the effects of carbohydrate and glutamine supplementation on salivary immunity after exercise at a simulated altitude of 4500 m. Fifteen volunteers performed exercise of 70% of VO_{2peak} until exhaustion and were divided into three groups: hypoxia placebo, hypoxia 8% maltodextrin (200 mL/20 min), and hypoxia after six days glutamine (20 g/day) and 8% maltodextrin (200 mL/20 min). All procedures were randomized and double-blind. Saliva was collected at rest (basal), before exercise (pre-exercise), immediately after exercise (post-exercise), and two hours after exercise. Analysis of Variance (ANOVA) for repeated measures and Tukey post hoc test were performed. Statistical significance was set at $p < 0.05$. SaO_2% reduced when comparing baseline vs. pre-exercise, post-exercise, and after recovery for all three groups. There was also a reduction of SaO_2% in pre-exercise vs. post-exercise for the hypoxia group and an increase was observed in pre-exercise vs. recovery for both supplementation groups, and between post-exercise and for the three groups studied. There was an increase of salivary flow in post-exercise vs. recovery in Hypoxia + Carbohydrate group. Immunoglobulin A (IgA) decreased from baseline vs. post-exercise for Hypoxia + Glutamine group. Interleukin 10 (IL-10) increased from post-exercise vs. after recovery in Hypoxia + Carbohydrate group. Reduction of tumor necrosis factor alpha (TNF-α) was observed from baseline vs. post-exercise and after recovery for the Hypoxia + Carbohydrate group; a lower concentration was observed in pre-exercise vs. post-exercise and recovery. TNF-α had a reduction from baseline vs. post-exercise for both supplementation groups, and a lower secretion between baseline vs. recovery, and pre-exercise vs. post-exercise for Hypoxia + Carbohydrate group. Five hours of hypoxia and exercise did not change IgA. Carbohydrates, with greater efficiency than glutamine, induced anti-inflammatory responses.

Keywords: supplementation; carbohydrate; hypoxia; physical exercise; glutamine; high altitude; innate immune response; oral mucosal immunity

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1. Introduction

Mucosal immunity, particularly in saliva, is considered the first line of defense against pathogens, because it contains numerous protective proteins. Some of these, such as salivary immunoglobulins (Igs), are involved in innate and adaptive immune responses [1]. In addition to Igs, there are also cytokines, such as interleukin (IL)-1ß, tumor necrosis factor (TNF)-α, and IL-6, that are used to assess the response to acute stress, stimulating immune cells, and modulating local inflammation [2,3].

Recent data suggest that exposure to hypoxia may modulate important aspects of innate immune responses [4], inflammation [5–7], and metabolism [8,9]. However, this issue has not been fully clarified, and only a few studies have been conducted under hypoxic conditions with the specific objective of investigating different immune/inflammatory parameters among humans [10].

It is known that exercise influences mucosal immunity, but the nature of this effect has not reached a consensus yet [11,12]. Some studies show that acute moderate-intensity exercise can result in a reduction of immunoglobulin A (IgA) concentration post-exercise; some do not describe any changes, while others report an increased concentration of IgA [13]. IgA is the most abundant protein in the antibacterial mucosal and it is considered the best indicator of oral mucosal immunity. Intense exercise causes a reduction in IgA levels [13] and increases inInterleukin-1 ß (IL-1ß), TNF-α, and IL-6 concentrations [2], resulting in poor performance of the immune function of the mucous membranes, increasing the incidence of upper respiratory tract infections (URTIs), and the emergence of other opportunistic diseases [14].

Thus, it is observed that exercise may modulate mucosal immunity under normal atmospheric pressure, but when exercise takes place in high altitude, it becomes a greater challenge for the body, since hypoxia and exercise are considered stressors that can act together. Evidence suggests that this combination may result in a more pronounced impact on the immune function of the oral mucosa and may trigger an intense immunosuppression [15,16].

On the other hand, studies have analyzed nutritional strategies that are efficient at sea level [17–20] to help mitigate the effects of exercise at altitude, providing better performance and prevention of infections [21].

When considering the anti-inflammatory effect of glutamine on stress factors, like exercise, harsh environments [21], and diseases (cancer, sepsis, burns, trauma) [22], this supplement can regress inflammation even during long-term exercises performed at sea level. Therefore, glutamine supplements have been shown to decrease the number of URTIs in athletes by promoting the production of IgA [23] and maintaining the balance of pro/anti-inflammatory markers [24].

On the other hand, carbohydrate supplements are used as a strategy to reduce the effects caused by the exercise on the immune system [25], and also contribute to improve performance. The intake of carbohydrate can significantly alter the immune response to intense exercise by attenuating the proliferation of lymphocytes, and by modulating cytokine pro/anti-inflammatory markers [26].

It has been shown that carbohydrate and glutamine supplements can be used isolated as a strategy to reverse the deteriorating mucosal immunity after strenuous exercise at sea level [23,27], however, the combined effect of both supplements is still not clarified.

In this context, we propose that the nutritional strategies used to prevent immune suppression after strenuous exercise at sea level can also be effective in hypoxic conditions. Thus, the objective of this study was to analyze the effect of carbohydrate and glutamine supplementation on oral mucosal immunity after exercise at a simulated altitude of 4500 m.

2. Methods

2.1. Experimental Design

This was a randomized, double-blind, placebo controlled crossover study and the sample size was determined using a statistics website from the Australian government [28]. After starting the clinical study, there were no changes in the methodology.

2.2. Participants

The sample of this study included 15 healthy male volunteers (women were not included in the sample to avoid the possible influences of female sex hormones) that were physically active (performing physical activity at least 3x/week for 90 min each session) with the following physiological and anthropometric characteristics: age: 26.4 ± 3.9 years old; body mass: 73.7 ± 8.7 kg;

height: 1.76 ± 0.02 m; Body Mass Index (BMI): 23.7 ± 2.5 kg/m^2; VO_{2peak}: 50.6 ± 5.4 mL/kg/min; maximum heart rate: 189.9 ± 8.2 beats per minute. Exclusion criteria were defined as: health problems; alterations in the electrocardiogram (ECG) at rest, stress and clinical evaluations, smoking, use of drugs, alcohol abuse, use of any medication that could interfere with the study results, and exposure to hypoxia during the previous six months. Figure 1 is a CONSORT flow diagram [29], explaining the stages of the randomized study. Initially, 60 volunteers were recruited to take part in the study, however 37 were eliminated based on the exclusion criteria. Of the 23 volunteers remaining, only 15 completed all the requirements.

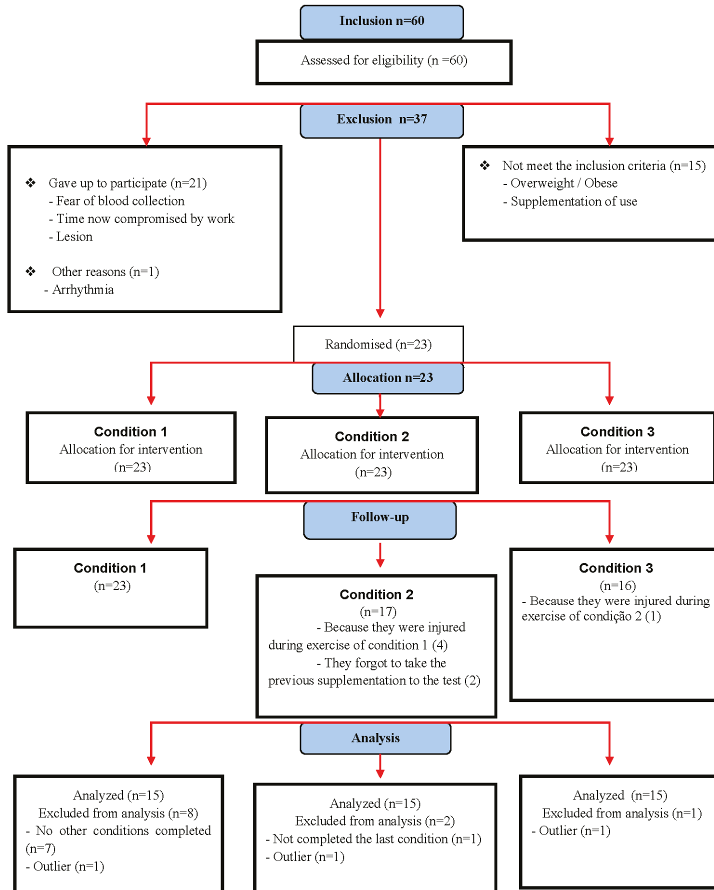

Figure 1. CONSORT flow diagram 2010.

Data were collected at the Interdisciplinary Laboratory for Exercise Physiology (LAIFE), Federal University of São Paulo (UNIFESP), São Paulo, between December 2014 and July 2015. The study procedures were approved by the Research Ethics Committee of the Federal University of São Paulo (Ethical approval code: 69 839/2014) on 4 March 2015 and are in accordance with the guidelines established by Resolution #466 of the Ministry of Health and the International Declaration of Helsinki.

2.3. Intervention

The participants came to the laboratory four times, with an interval of six days between each visit. During the first session, relevant information was presented, which consisted of objectives, procedures, guidelines for not taking supplements, and only low-intensity exercise. The participants were randomized into three groups [30] and were asked to sign a consent form. Next, they performed resting ECG, stress and cardiopulmonary exercise tests. The blinding process occurred in order to offer supplements and placebos that had the same characteristics of color, consistency, smell, taste, and presentation. An individual, oblivious of the study, was responsible for delivering the supplements to the participants every week, so the researches had no contact with the supplements. During the next three visits, the participants performed three random, blinded exercise sessions:

1. Group Hypoxia (Exercise + Altitude + Placebo): Participants consumed glutamine placebo supplements during the six days prior to the test (10 g corn starch + 10 g lactose), taken in the evening. During test day, they performed an exercise session at 70% of VO_{2peak} at a simulated altitude of 4500 m and were given a carbohydrate placebo supplement (Crystal Light®—Kraft Foods, Inc. strawberry, Chicago, IL, USA), 200 mL every 20 min during exercise and during recovery for two hours.
2. Group Hypoxia + CHO (Exercise + Altitude + Carbohydrate): Participants consumed glutamine placebo supplements during the six days prior to the test (10 g corn starch + 10 g lactose) taken in the evening. During test day, they performed an exercise session at 70% of VO_{2peak} at a simulated altitude of 4500 m, and were given carbohydrate supplements (Maltodextrin strawberry flavor—Probiótica®—Laboratories, Embu das Artes, São Paulo, Brazil), 200 mL at a concentration of 8% every 20 min during exercise and during recovery for two hours.
3. Group Hypoxia + GLN (Exercise + Altitude + Carbohydrate + Glutamine): Participants consumed 20 g of glutamine (Probiótica®—Laboratories, Embu das Artes, São Paulo, Brazil) in the six days prior to the test, between 8:00–10:00 p.m. During test day, they performed an exercise session at 70% of VO_{2peak} at a simulated altitude of 4500 m, and were given carbohydrate supplements (Maltodextrin strawberry flavor—Probiótica®—Laboratories, Embu das Artes, São Paulo, Brazil), 200 mL at a concentration of 8% every 20 min during exercise and during recovery for two hours.

For all exercise sessions, water intake was ad libitum. However, there was no control of the ingested volume.

Determination of VO_{2peak}

To determine the VO_{2peak} in normoxic conditions, a test was performed with progressive intensity on a treadmill (LifeFitness®- 9700HR, Rosemont, IL, USA) with an initial speed of 7 km/h and increase of 1 km/h every minute until exhaustion (defined as the incapacity to keep up with the speed of the treadmill for 15 s or until the volunteer requested to stop the test after being encouraged to continue [31]) The encouragement for the volunteers was similar in all tests and carried out by the same person. During the test, we used a fixed inclination of 1% to simulate the physical stress of field tests [32].

Heart rate was monitored with a Polar Vantage NV watch (*Polar*®, Sark Products, Waltham, MA, USA), blood pressure was monitored by sphygmomanometer and stethoscope, and perceived exertion by the Borg scale (6 to 20) [33]. The respiratory parameters were measured by a gas analyzer (Cosmed Quark PFT model, Albano Laziale, Rome, Italy), pulmonary function (FRC & DLCO, Albano Laziale, Rome, Italy) was analyzed using a facemask (Hans Rudolph Inc., Shawnee, KS, USA). All calibration procedures were performed according to the manufacturer's recommendations.

2.4. Altitude Simulation

A normobaric chamber was used (normobaric chamber CAT—Colorado Altitude Training™/CAT-12 Air Unit®, Lousiville, CO, USA) to simulate an altitude of 4500 m (changing carbon

dioxide and oxygen concentrations (equivalent to a barometric pressure of 433 mmHg and a fraction of inspired oxygen of 13.5% O_2)).

2.5. Sessions of Exercise and Recovery

The participants spent the first two hours in the hypoxic chamber at rest and then began to exercise on a treadmill (LifeFitness®- 9700HR, Rosemont, IL, USA) with a fixed inclination of 1% and intensity of 70% of VO_{2peak} until exhaustion or up to one hour. After exercising, they remained in the chamber for two more hours for recovery. Each test was followed by six days of rest, which was considered long enough to eliminate the effects of hypoxia [34] and supplementation [24]. All exercise session were performed after an overnight of fasting to avoid possible influences of diet and to maintain a standardized metabolic condition. Testing began at 7:30 a.m. to avoid circadian influences.

2.6. Hemoglobin O_2 Saturation (SaO_2%)

During all tests, the SaO_2% was monitored by a pulse oximeter on the finger (FingerPulse®, MD300C202 model, Beijing, China) and assessed during four stages with saliva collection.

2.7. Saliva Collection

The saliva samples were collected using the Salivet method (cylindrical roller bearings that absorb saliva during the period of a minute) during four moments: immediately before entering the chamber (baseline), immediately before starting exercise (pre-exercise), immediately after exercise (post-exercise), and after two hours of recovery (after 2:00). After collection, the sample was put into a tube and centrifuged at a speed of $600\times\ g$ for 20 min. Then, a clear fluid specimen was obtained and stored frozen (−80 °C) for analysis.

2.8. Determinants in Saliva

IgA was determined by immunoturbidimetric method using Kits from Labtest® (Lagoa Santa, MG, Brazil) and cytokines (TNF-α, IL-6, and IL-10) were determined using Milliplex Kits® (Darmstadt, Germany).

The flow rates of IgA, TNF-α, IL-6, and IL-10 were calculated by multiplying the concentration of each parameter by salivary flow (mL/min) as described by Usui et al. (2011) [2].

2.9. Statistical Analysis

Data normality was verified by the Shapiro-Wilk test. Descriptive analysis consisted of mean and standard error. ANOVA for repeated measures followed by post hoc Tukey test verified the interactions between groups and time, and Cohen's d was calculated to estimate effect size: 0.20–0.30 = small effect size; 0.40–0.70 = medium effect size, and ≥0.80 = large effect size. The software Statistics® 7.0 (StatSoft, Inc., Tulsa, OK, USA) was used for the statistical analyzes and the level of significance was set at $p < 0.05$.

3. Results

The results are presented in tables and figures. There was no significant difference between groups Hypoxia, Hypoxia + Carbohydrate, and Hypoxia + Glutamine for SaO_2% ($F = 2.2$, $p = 0.119$), as shown in Table 1. However, significant differences were observed regarding time ($F = 248.5$, $p < 0.001$). When comparing the moment of measurement, we found reduction of SaO_2% at baseline versus pre-exercise ($p < 0.001$) and baseline versus post-exercise ($p < 0.001$). Additionally, there was reduction at baseline in relation to after recovery for all three groups ($p < 0.01$). There was also a reduction of SaO_2% in pre-exercise versus post-exercise ($p < 0.001$) for the hypoxia group. However, an increase was observed in pre-exercise compared to recovery for both groups with supplements ($p < 0.001$), and between post-exercise and recovery for all groups ($p < 0.001$). There was significant difference in

the interaction between the groups and time (F = 5.5, $p < 0.001$), with an increase in Hypoxia group compared to Hypoxia + Glutamine group in recovery ($p = 0.02$).

Table 1. O_2 saturation percent (SaO_2%).

		Condition		
		Hypoxia	**Hypoxia + CHO**	**Hypoxia + GLN**
SaO_2%	Basal	97.13 ± 0.27	97.13 ± 0.21	96.87 ± 0.24
	Pre-exercise	85.47 ± 1.35 ^{A}	82.40 ± 1.23 ^{A}	84.33 ± 1.01 ^{A}
	Post-exercise	79.67 ± 1.37 ^{AB}	81.47 ± 1.02 ^{A}	81.53 ± 1.43 ^{A}
	2 h after	85.40 ± 1.00 ^{AC}	89.33 ± 0.46 ^{ABC}	90.27 ± 0.55 ^{ABC*}

The results of SaO_2 (%) were described by mean ± Standard Error (SE). The interactions of group versus time was Analysis of Variance (ANOVA) for repeated measures followed by Post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. ^{A} statistically significant in relation to basal. ^{B} statistically significant in relation to pre-exercise. ^{C} statistically significant in relation to post-exercise. * Statistically significant in relation to hypoxia condition. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

Figure 2 shows results of time to exhaustion. There were no statistical differences regarding time to exhaustion between Hypoxia group (27.6 ± 5.46), Hypoxia + Carbohydrate group (29.2 ± 6.49), and Hypoxia + Glutamine group (23.66 ± 4.95).

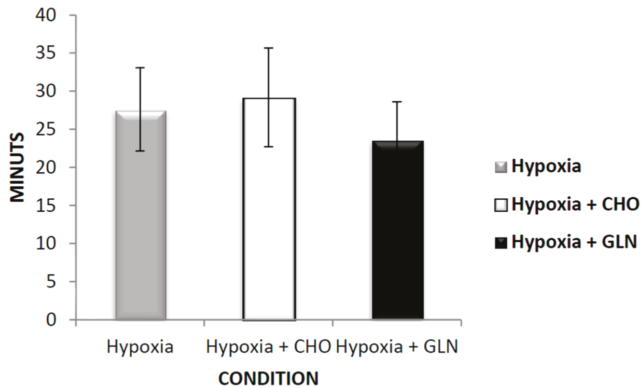

Figure 2. The results of time of exhaustion (min) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

Salivary flow (mL/min) results are presented in Table 2. No differences were observed between groups (F = 0.78, $p = 0.925$), but significant difference was found regarding time (F = 7.927, $p < 0.001$). There was an increase of salivary flow post-exercise versus recovery ($p < 0.001$) for the Hypoxia + Carbohydrate group. In the interaction of groups versus time, there was no significant difference (F = 0.863, $p = 0.524$).

Table 2. Salivary Flow.

		Condition		
		Hypoxia	Hypoxia + CHO	Hypoxia + GLN
SaO_2%	Basal	0.90 ± 0.11	0.89 ± 0.12	0.89 ± 0.09
	Pre-exercise	0.88 ± 0.11	0.81 ± 0.10	0.76 ± 0.08
	Post-exercise	0.75 ± 0.10	0.76 ± 0.11	0.79 ± 0.13
	2 h after	0.92 ± 0.11	1.04 ± 0.14 ^{C}	0.85 ± 0.10

The results of Salivary Flow (mL/min) were described by mean ± Standard Errros (SE). The interactions of group versus time was Analays of Variance (ANOVA) for repeated measures followed by Post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. ^{C} statistically significant in relation to post-exercise. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

Regarding immunity, we measured IgA and cytokines. The salivary concentration of IgA showed no difference between groups (F = 0.080, $p = 0.923$), time (F = 2.578, $p = 0.057$), and interaction (F = 1.133, $p = 0.347$). However, Cohen's effect size d for the concentration of salivary IgA of pre-exercise versus post-exercise was 0.9 and pre-exercise versus recovery was 2.27 for the Hypoxia group; for the Hypoxia + Carbohydrate group, pre-exercise versus recovery showed effect size d = 1.06, and baseline versus post-exercise d = 1.9. The Hypoxia + Glutamine group showed effect size d = 2.6 for baseline versus recovery (Table 3). The Cohen's effect size d > 0.08 is considered a high effect. IgA secretion rate presented no differences between groups ($F = 0.074$, $p = 0.929$). However, a significant difference was observed for time (F = 6.462, $p < 0.001$), and a reduction was found from baseline versus post-exercise ($p < 0.001$) for the group Hypoxia + Glutamine. The Hypoxia group showed a reduction of 22.5% post-exercise compared to baseline, with no statistical difference, but Cohen's effect size d of 1.58. There was no interaction of groups versus time ($F = 1.262$, $p = 0.280$) (Figure 3).

Table 3. IgA, IL-10, TNF-α e IL-6 Concentration

		Condition		
		Hypoxia	Hypoxia + CHO	Hypoxia + GLN
IgA	Basal	48.40 ± 2.07	46.47 ± 1.6	49.80 ± 2.27
	Pre-exercise	49.93 ± 2.21	50.47 ± 3.30	48.40 ± 1.67
	Post-exercise	47.33 ± 3.34	50.80 ± 3.88	45.60 ± 2.02
	2 h after	45.07 ± 1.25	46.73 ± 3.70	47.00 ± 2.60
IL-10	Basal	1.11 ± 0.15	1.22 ± 0.16	1.13 ± 0.17
	Pre-exercise	1.14 ± 0.17	1.27 ± 0.19	1.25 ± 0.20
	Post-exercise	1.08 ± 0.12	1.01 ± 0.13	1.02 ± 0.17
	2 h after	1.11 ± 0.16	1.22 ± 0.15	1.24 ± 0.15
TNF-α	Basal	2.06 ± 0.80	2.07 ± 0.69	3.69 ± 1.79
	Pre-exercise	2.18 ± 0.67	2.30 ± 0.80	2.74 ± 0.75
	Post-exercise	2.02 ± 0.98	1.54 ± 0.62 ^{AB}	3.06 ± 2.25
	2 h after	1.34 ± 0.44	1.12 ± 0.39 ^{AB}	2.03 ± 0.73
IL-6	Basal	1.05 ± 0.26	1.50 ± 0.68	1.42 ± 0.58
	Pre-exercise	0.93 ± 0.22	1.57 ± 0.51	1.92 ± 0.63
	Post-exercise	0.97 ± 0.30	1.15 ± 0.36	1.26 ± 0.48
	2 h after	0.82 ± 0.22	1.12 ± 0.37	1.06 ± 0.37

The results of concentration of Immunoglobulin A (IgA) (mg/dL), Interleukin-10 (IL-10), Tumoral Necrosis Factor-α (TNF-α) e Interleukin-6 (IL-6), in pg/mL were described by mean ± Standard Error (SE). The interactions of group versus time was Analaysis of Variance (ANOVA) *for repeated measures* followed by *Post hoc of Tukey* test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. ^{A} statistically significant in relation to basal. ^{B} statistically significant in relation to pre-exercise. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

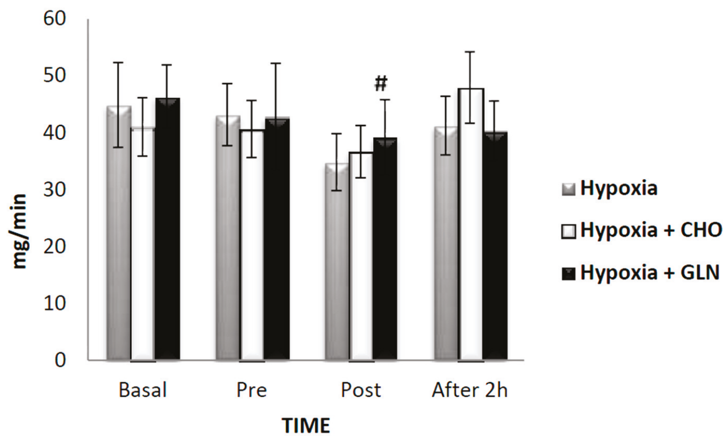

Figure 3. The results of secretory immunoglobulin A (IgA) (mg/min) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. # Statistically significant in relation to basal. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

Results related to pro- and anti-inflammatory cytokines are presented in Table 3. Regarding IL-10 concentration, there was no significant differences between groups ($F = 0.148$, $p = 0.863$), time ($F = 0.893$, $p = 0.447$), and interaction ($F = 0.487$, $p = 0.817$). Cohen's effect size d for all comparisons related to IL-10 was below 0.2. This is considered a small effect size.

Regarding the rate of saliva secretion with IL-10 (Figure 4), there were no significant differences between the three groups ($F = 0.170$, $p = 0.844$), but there was a significant difference in time ($F = 6.119$, $p < 0.001$). Among these differences, there was an increase between post-exercise versus recovery ($p < 0.001$) for Hypoxia + Carbohydrate group. There was no interaction for this parameter ($F = 0.589$, $p = 0.739$).

The salivary concentrations of TNF-α (Table 3) showed no difference between the three groups ($F = 0.48$, $p = 0.624$). Regarding time, a reduction was observed ($F = 15.88$, $p < 0.001$) from baseline versus post-exercise ($p = 0.001$) and after recovery ($p < 0.001$) for the Hypoxia + Carbohydrate group. Similarly, a lower concentration was observed in pre-exercise time versus post-exercise ($p < 0.001$) and recovery ($p < 0.001$). There was no interaction of groups versus time ($F = 1.61$, $p = 0.148$).

When considering the rate of saliva secretion of TNF-α (Figure 5), there was no significant difference between the three groups ($F = 0.33$, $p = 0.723$). However, there were differences in time for TNF-α secretion rate ($F = 14.63$, $p < 0.001$). Among these differences, we observed a reduction from baseline versus post-exercise ($p < 0.001$) for both supplemented groups, and a lower secretion of baseline compared to recovery ($p < 0.001$), and pre-exercise versus post-exercise ($p < 0.001$) for Hypoxia + Carbohydrate group. There was no interaction for this parameter ($F = 3.01$, $p = 0.408$).

Table 3 shows the salivary concentration of IL-6 and Figure 6 shows the salivary secretion rate of IL-6. Table 3 and Figure 4 showed no significant differences between groups ($F = 0.213$, $p = 0.809$) and ($F = 0.138$, $p = 0.872$), time (F = 3.200, $p = 0.02$) and ($F = 2.555$, $p = 0.05$), and interaction (F = 0.726, $p = 0.629$) and ($F = 0.600$, $p = 0.730$), respectively.

The ratio of salivary secretion rate of TNF-α/IL-10 is presented in Figure 7 and showed differences between groups ($F = 5.2$, $p < 0.001$), with an elevation of Hypoxia + Carbohydrate versus Hypoxia + Glutamine ($p = 0.01$). A significant difference was observed for time ($F = 608.0$, $p < 0.001$), and the reduction was found at baseline versus pre-exercise ($p < 0.001$), post-exercise ($p < 0.001$), and recovery ($p < 0.001$) in the group Hypoxia + Carbohydrate and Hypoxia + Glutamine. There was interaction

of groups versus time ($F = 4.6$, $p = 0.001$), including an elevation between baseline in Hypoxia group compared to Hypoxia + Glutamine group ($p = 0.01$), and baseline in Hypoxia + Carbohydrate group compared to Hypoxia and Hypoxia + Glutamine group ($p = 0.01$).

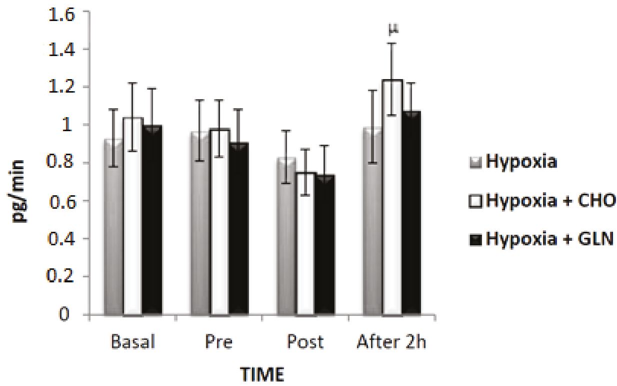

Figure 4. The results of interleukin (IL)-10 secretory (pg/min) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. μ statistically significant in relation to post-exercise. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

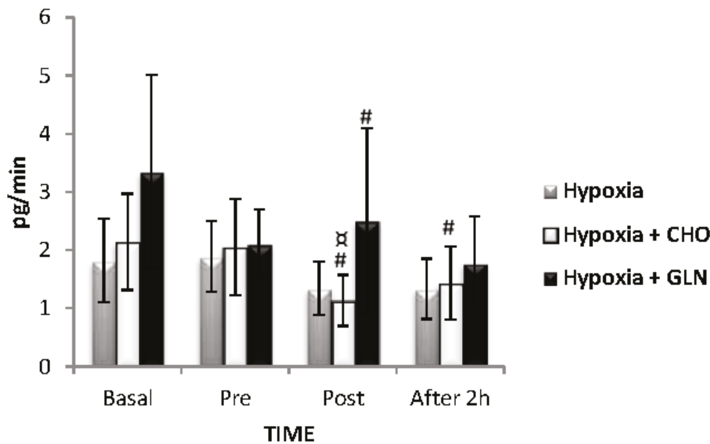

Figure 5. The results of tumor necrosis factor (TNF)-α secretory (pg/min) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. # Different in relation toBasal. ¤ Statistically significant in relation to pre-exercise. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

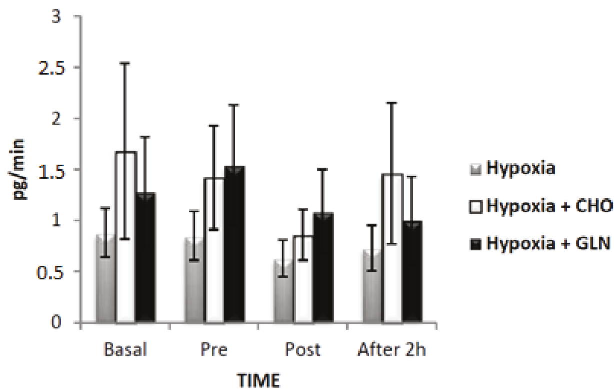

Figure 6. The results of IL-6 secretory (pg/min) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

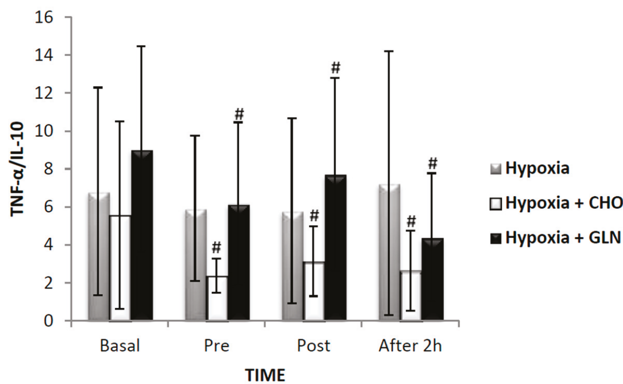

Figure 7. The results of salivary secretion rate of Tumor necrosis factor- α/Interleukin-10 (TNF-α/IL-10) was described by mean ± Standard Error (SE). The interaction of group versus time was analyzed by Analysis of Variance (ANOVA) for repeated measures followed by post hoc of Tukey test. The level of significance was set at $p < 0.05$. $n = 15$ volunteers. # Different in relation to Basal. Hypoxia + CHO = Hypoxia + carbohydrate and hypoxia + GLN = hypoxia + glutamine.

4. Discussion

The aim of this study was to analyze the effect of carbohydrate and glutamine supplementation on oral mucosal immunity after exercise at a simulated altitude of 4500 m. The main finding of this study was that strenuous exercise associated with hypoxia, with or without supplementation, did not change salivary IgA. Despite the decrease in the pro/anti-inflammatory balance, an anti-inflammatory response was found in the group with carbohydrate supplementation because of changes in IL-10 and TNF-α concentrations.

According to several studies conducted at sea level, carbohydrate and/or glutamine supplementation have shown to be effective on mitigating the stress effects of vigorous exercise on the immune system. Taking into consideration that the number of people that travel to places of high altitudes for tourism, work, and sports increases each year, it becomes of great importance to

elucidate the effects of carbohydrate and/or glutamine supplementation in hypoxic environments on the oral mucosal immunity, which is considered a practical method to indicate stress. Therefore, in the future, new interventions may be proposed and designed to minimize the effects of hypoxia among athletes, travelers, workers, and people chronically exposed to high altitudes.

Regarding results involving SaO_2%, the values at baseline were not significantly different for the groups, since they are in normal oxygen concentration. However, a reduction in SaO_2% was found after two hours of exposure for all groups, proving the efficiency of the hypoxia model used in this study and confirming the results found in the studies conducted by Tannheimer et al. [35], Mazzeo [14], and Pomidori et al. [36]. However, after two hours of recovery in hypoxia, SaO_2% increased almost immediately post-exercise, suggesting recovery. However, this was not enough time to restore the values to baseline levels.

The SaO_2% results of the Hypoxia + Carbohydrate group were similar to the group with no supplementation at baseline, but different after two hours of recovery with an increase in SaO_2% compared to pre-exercise and post-exercise. Such modifications are related to carbohydrate intake, increasing the concentration of CO_2 to a level that stimulates ventilation, thus enhancing blood oxygenation and reducing the desaturation of hypoxia [37,38]. The Hypoxia + Glutamine group showed similar changes compared to the Hypoxia group at baseline, although the results were different after two hours of recovery, showing an increase at pre-exercise, post-exercise, and hypoxic condition. The reasons for the restoration of SaO_2% in the Hypoxia + Glutamine group are not known, but it is suggested that the increased availability of plasma glutamine may interfere with the central synthesis of glutamate, an excitatory neurotransmitter that stimulates ventilation [39], and thus contribute to SaO_2% recovery.

Despite not evaluating plasma concentration of glutamine, a previous study with a similar protocol showed a 65.8% increase of glutamine post-exercise when compared to pre-exercise [24], reinforcing our hypothesis. Another fact to consider in the Hypoxia + Glutamine group is the combined action of the two supplements [40], contributing to increased ventilation in different pathways [37,39].

Pilardeau et al. [41] were the first to describe salivary flow in hypoxia; today it is known that salivary secretion can be affected by neural control of the autonomic nervous system, which indirectly regulates salivary flow and saliva composition [13]. The stress of intense exercise added to hypoxic environment stimulates the sympathetic nervous system, contracting blood vessels in salivary glands, which leads to a reduction in flow rate [27]. Our results are partly explained by these mechanisms, showing that salivary flow in hypoxic condition reduced 17% from baseline. However, the intake of both supplements appears to alleviate this effect, since the Hypoxia + Carbohydrate group showed a reduction of only 14% in salivary flow post-exercise compared to baseline. Interestingly, after two hours of recovery, supplementation with carbohydrates was able to promote a significant increase of 27% in flow compared to the end of the exercise. Bishop et al. [27] analyzed participants in normoxic conditions after two hours of riding a bicycle at 60% of VO_{2max} and found that consumption of carbohydrates (60 gL) increased salivary flow one hour after exercise, probably due to a reduction of sympathetic/parasympathetic balance [1].

The changes in salivary flow during and after exercise directly affect the concentration of salivary IgA [13,42], however, our results showed that the stimulation of salivary flow followed by the stress of exercise and hypoxia was not able to promote changes of total IgA concentration in any of the conditions. These results are similar to a study by Svendsen et al. [43] that analyzed participants exposed to hypobaric hypoxia conditions equivalent to 2000 m during 75 min of cycling at 70% VO_{2peak}. This finding may have occurred due to the high intensity of exercise and its immunosuppressive function, preventing the elevation of IgA [44], or because the reduced time of exposure to hypoxia was not enough to modify secretion of IgA [1].

The IgA secretion rate in the Hypoxic group was reduced by 22.5% post-exercise compared to baseline; statistically this reduction was not significant, but it can be physiologically important, since IgA is the most abundant protection protein in saliva [1]. The lower level of IgA secretion

indicates a specific reduction in the synthesis and/or secretion of salivary IgA in response to stress created by intense exercise [18,45] coupled with hypoxia. When supplements were taken by the participants, this reduction was slightly smaller (i.e., 10.6% in the Hypoxia + Carbohydrate group and 15.5% in the Hypoxia + Glutamine group). Our findings are similar to the study conducted by Krzywkowski et al. [45], involving normoxia with glutamine supplementation (17.5 g), which showed the same tendency of exercise (two hours of bicycle exercise at 75% VO_{2max}) to reduce salivary IgA during and up to two hours after exercise.

The production of IgA in saliva may be mediated by several factors, such as stress hormones, nutritional factors, circadian cycle, hydration, alcohol intake, and also cytokines [1,2,13,46]. The effects of exercise on the production of cytokines in saliva are not well understood [2], especially in hypoxia [21]. Therefore, the present study was the first to investigate the effect of carbohydrate and glutamine supplementation on concentration of cytokines after exercise in hypoxic condition.

The concentration of IL-10 and its secretion rates were not different when comparing time for any of the groups. However, despite the secretion rate being slightly lower in post-exercise when compared to baseline, we found that supplementation with carbohydrates was able to increase IL-10 after two hours of recovery, showing that the anti-inflammatory role of carbohydrate [46] can also be observed in saliva, thereby contributing to the maintenance of homeostasis at this site and helping to preserve mucosal immune responses [47]. We found similar results in the Hypoxia + Glutamine group, suggesting that, despite the increase of IL-10 by approximately 25% post-exercise compared to baseline, glutamine supplementation was not able to modulate the pro/anti-inflammatory balance by modification of IL-10.

The concentration and rate of secretion of TNF-α did not change in the Hypoxia group, however, in normoxic conditions, an increase of TNF-α was found in saliva during and after intense exercise [2], and a reduction after one-hour of recovery [48]. When assessing the Hypoxia + Carbohydrate group, TNF-α decreased after exercise and recovery, suggesting that the increase of IL-10 in saliva, mediated by supplementation with carbohydrates, may be responsible for a decrease in TNF-α secretion rate, similar to what occurs in other tissues, and enhancing the anti-inflammatory role of carbohydrates. In fact, supplementation with carbohydrates was able to attenuate the inflammatory process promoted by exercise and hypoxia, and modulated the balance between pro- and anti-inflammatory cytokines in saliva, as in normoxic conditions [49].

Regarding glutamine supplementation, we observed a decrease in TNF-α secretion rate immediately after exercise, and the reduction was more evident at the end of the second hour of recovery. These results demonstrated the anti-inflammatory role of glutamine in saliva, which has been observed in other tissues, directing the pro-inflammatory/anti-inflammatory balance toward an anti-inflammatory response [21,22].

The salivary concentration and secretion rate of IL-6 did not change in any of the groups. There are no results in the literature showing the effect of exercise on salivary IL-6 in hypoxia. Our results contradict the findings of Usui et al. [2], who observed an increase of IL-6 levels in saliva in normoxic conditions during and after exercise at 75% of $VO_{2max,}$ including 80 min post-exercise. Thus, we cannot suggest what mechanisms are responsible for regulating IL-6 in hypoxia, but they are probably different from those in normoxic condition (i.e., maintaining homeostasis of blood and hepatic glucose and stimulating the release of C Reactive Protein (CRP). We believe this finding is a reflection of the increased use of IL-6 in its hematopoietic purpose [50,51] because it causes almost immediate reduction of SaO_2% and deterioration of O_2 due to hypoxia.

5. Conclusions

We conclude that five hours in hypoxia associated with strenuous exercise was not enough to promote a change in salivary IgA. Supplementation with carbohydrates and glutamine produced changes in the pro/anti-inflammatory balance, stimulating an inflammatory response in oral mucosa.

However, our results should be interpreted with caution in regards to their generalizability because we only assessed male subjects.

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Author Contributions: Aline Venticinque Caris conceived and designed the experiments, analyzed the data, and wrote the paper; Edgar Tavares Da Silva and Samile Amorim Dos Santos performed the experiments; Sergio Tufik contributed reagents/materials/analysis tools; Ronaldo Vagner Thomatieli Dos Santos conceived and designed the experiments and wrote the paper.

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8

Vitamin D and Weight Cycling: Impact on Injury, Illness, and Inflammation in Collegiate Wrestlers

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Abstract: This study explored the link between vitamin D status and frequency of skin infections, inflammation, and injury in college wrestlers during an academic year. Methods: Serum 25-hydroxyvitamin D (25(OH)D) (n = 19), plasma cytokine (TNF-α, IL-6, IL-10) (n = 18) concentrations, and body weight/composition were measured and injury/illness/skin infection data were collected in fall, winter, and spring. Results: In the fall, 74% of wrestlers had vitamin D concentrations <32 ng/mL which increased to 94% in winter and spring. Wrestlers lost an average of 3.4 $\pm$ 3.9 kg ($p < 0.001$) during the season with corresponding decreases in fat mass and increases in lean mass ($p < 0.01$). An inverse association between 25(OH)D concentrations and total body mass and body fat percentage was observed at all-time points ($p < 0.01$). Concentrations of cytokines were highly variable among individuals and did not change across time ($p > 0.05$). Correlations between vitamin D status, cytokines, or frequency of illness, injury, or skin infections were not observed. Conclusions: A high prevalence of vitamin D insufficiency (<32 ng/mL) and deficiency (<20 ng/mL) was observed in wrestlers and was associated with higher adiposity. It remains unclear if higher vitamin D status would reduce injury, illness, and skin infection risk.

Keywords: vitamin D; wrestling; exercise; athletes; inflammation

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1. Introduction

Increasing evidence has linked low vitamin D status to a variety of health conditions including osteoporosis, cardiovascular disease, diabetes, depression, multiple sclerosis, rheumatoid arthritis and certain types of cancer [1–3]. Research has also observed a link between low vitamin D status and increased susceptibility of upper respiratory tract infections (URTI) [4] including influenza and the common cold [4,5]. Even though vitamin D is considered a vitamin, it is unique in that it both acts as a hormone, assisting in regulation of serum calcium, and may be obtained from dietary and endogenously synthesized sources. Endogenous synthesis occurs in the skin with exposure to sufficient ultra violet B (UVB) light [1,6] during peak hours (10 a.m. to 2 p.m.). Vitamin D cannot be made in the winter months at distances greater than 37 degrees north or south [2]. Natural or fortified dietary sources include fatty fish, whole milk, and some brands of yogurt, margarine, fruit juice, and ready-to-eat cereals [2]. Although vitamin D deficiency has been considered a nutritional problem

of the past, it has re-emerged as a public health concern. In fact, some researchers believe it is an unrecognized epidemic in adults lacking exposure to adequate sunlight [1].

In athletic populations, vitamin D may be important for optimal health and performance. Vitamin D deficiency has been associated with reduced strength [7], prolonged recovery from surgery [8], altered inflammatory markers [9,10], and increased risk for injury and illness [11–13] in both athletes and non-athletes. In athletes, the prevalence of vitamin D deficiency and insufficiency varies by sport, training location, skin color [13], adequate sunlight exposure between 10 a.m. and 2 p.m., is more prevalent among athletes who train indoors versus outdoors [11,14,15], and who have higher body fat percentages [16]. A summary of vitamin D status documented in athletes and active individuals is outlined in Table S1. Vitamin D status is generally lower in the winter and among athletes who train predominantly indoors versus outdoors and who live at higher latitudes (i.e., >37 degrees North or South). In addition, serum concentrations tend to decline between fall and spring, and supplementation may or may not be effective for reaching optimum concentrations depending on dosage [17]. Current studies are inconsistent as to whether or not having higher vitamin D status translates to improved performance measures [18].

Wrestlers, particularly at the college level, are at risk for skin infections from mat and skin-to-skin contact and may be more prone to both compromised immune function and poor vitamin D status due to nutrient restriction from cutting weight [19–21], indoor training, and competing during a season of limited sun exposure (October-March) when URTI risk is elevated [11,22]. Although the vitamin D status of collegiate athletes has previously been evaluated, little is known about the status of wrestlers who may be at increased risk for deficiency/insufficiency due to indoor training and chronic dietary restriction and weight cycling. Therefore, the purpose of this study was to assess vitamin D status of male college wrestlers during the academic year and determine if low vitamin D status (i.e., low circulating concentrations of 25-hydroxy vitamin D) was associated with documented incidence of acute illness, including skin infections, and with circulating pro-inflammatory cytokines interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) and the anti-inflammatory cytokine interleukin-10 (IL-10). A secondary purpose was to explore whether weight and body composition changes further impacted seasonal changes in vitamin D concentration and cytokine concentrations. We hypothesized that wrestlers with suboptimal vitamin D status (25(OH)D <32 ng/mL) would have a higher incidence of acute illness including URTI and skin infections, higher pro-inflammatory markers, and lower anti-inflammatory markers compared to wrestlers with optimal vitamin D status (25(OH)D >40 ng/mL).

2. Materials and Methods

2.1. Study Design

This study took place during the 2014–2015 college academic year. All University of Wyoming (UW) athletes $\geq$18 years on UW's National Collegiate Athletic Association (NCAA) Division I wrestling team (n = 25) were invited to participate. Participation, however, was not required and the coaching staff received no information on which wrestlers participated in the study so as to avoid potential bias during training or competition. The study was approved by the UW Institutional Review Board (approval code #20140703JB00477, 3 July 2014) with written, informed consent obtained prior to participation.

2.2. Study Overview

Blood was drawn three times during the academic year (September, January, and April) in accordance with seasonal training and typically occurred in the morning after an overnight fast. Athletes were instructed to avoid exercise for at least 12 h prior to the draw. Height and weight were measured and a short questionnaire, administered at these same time points was used to assess vitamin D intake and lifestyle factors which may impact vitamin D status (i.e., estimated time spent outdoors,

sunscreen use, use of tanning beds, etc.) [11]. The vitamin D questionnaire was administered in a private setting and took approximately 10 min to complete [11]. Body composition was analyzed by dual energy X-ray absorptiometry (DXA) and occurred no more than 7 days away from the blood draw. Selected information contained within the athletes' medical charts, including illness and infection history (i.e., upper respiratory tract infections, gastritis, skin infection, etc.), illness progression, prescribed medications, and supplement use was obtained from medical records documented by the Sports Medicine staff as part of routine care.

2.3. Blood Analysis

Blood samples were appropriately post-processed and kept frozen at −20 degrees Celsius until analysis. 25(OH)D concentration was evaluated by Diasorin 25(OH)D radioimmunoassay (Bruce Hollis' Laboratory, the Medical University of South Carolina, Charleston, SC, USA). Inflammatory markers (TNF-α, IL-6, IL-10) were analyzed via enzyme-linked immunosorbent assay (ELISA) (QIAGEN, Santa Clarita, CA, USA) according to manufacturer's instructions. For cytokine analysis, plasma samples were thawed immediately before being assayed and vortexed for approximately 5 s to ensure adequate mixing. Absorbance was read at 450 nm using a standard ELISA microplate reader. Standards were diluted using a 1:2 dilution series. The eight standards of known cytokine concentrations included in each assay ranged from 15–2000 pg/mL. All samples were analyzed in duplicate. A log transformation was used to generate a regression equation to predict cytokine concentrations, reported as pg/mL.

2.4. Body Composition

Body composition was assessed via DXA. Subjects were instructed to avoid food and exercise at least two hours prior to testing. When possible, testing was done first thing in the morning (6:00–9:00 a.m.) after an overnight fast and abstaining from training for at least 12 h. Body mass (kg), fat mass (kg), fat free mass (kg), and body fat percentage were utilized for statistical analysis.

2.5. Weight Collection

Weekly weights were obtained from coaching staff from 3 September to 28 February. Wrestlers were weighed on the same scale, with minimal clothing, in their locker room prior to afternoon practice on each Monday by one of the wrestling coaches. In order to calculate the amount of weight lost each week to "make weight" for competition, each athlete's weight class was subtracted from his recorded Monday weight with the assumption that he would have to lose that amount in order to compete at the end of the week (Friday/Saturday). Because heavyweights are less likely to partake in traditional weight cutting practices, heavyweights were omitted from statistical analyses addressing weekly and seasonal weight changes. These weights were also utilized to calculate average weekly weight changes (Monday to Monday).

2.6. Injury, Illness, and Skin Infection

As part of routine medical care, injury, illness, and skin infection data were collected by the team physician and documented in student-athlete medical charts. In addition to documentation done by the physician, a certified athletic trainer (ATC) assigned to the sport performed daily skin checks and was present at each training session. The daily skin check allowed wrestlers to report any unusual formations on the skin.

2.7. Statistical Procedures

A sample size calculation for a descriptive study of a continuous variable (i.e., 25(OH)D concentration) was conducted using the average standard deviation for college athletes participating in indoor sports during the fall (8.2 ng/mL) and winter (5.6 ng/mL) from pervious data [11].

Using a 95% confidence interval (CI), an n = 19 to 41 was estimated for a total desired CI width of 5 and a sample of 10 to 21 was needed for a total CI width of 7 [23]. Correlation sample calculations for previously identified associations between 25(OH)D and fat mass ($r = -0.42$) [16], frequency of URTI ($r = -0.40$) [11] and TNF-α concentration ($r = -0.63$) [24] using previous coefficients as the expected correlation coefficient (one-tailed $\alpha = 0.05$ and $\beta = 0.20$) estimated that a sample size of n = 14 to 37 would be needed to determine whether correlation coefficients differed from zero. Given these calculations, efforts were placed on recruitment and longitudinal retention of as many of our total population of wrestlers of n = 25.

Data were analyzed using IBM SPSS Statistics 23 software. Repeated measures analysis of variance (ANOVA) was utilized to assess the change in body weight/body composition, serum 25(OH)D, and plasma cytokine concentrations over time. Correlation coefficients (Pearson's) were used to evaluate the associations among 25(OH)D and body weight/adiposity, weight change during the season, dietary and supplemental vitamin D intake (including vitamin D and calcium), frequency of infection and illness, and cytokine concentrations. Additional multilinear regression modeling was used to evaluate the association between vitamin D status and body weight/body composition adjusting for weight cycling pattern, weight class, age, athletic eligibility year, and other significant predictors (based on analysis of simple correlation coefficients). Spearman's rank correlations were used to identify intra-cytokine association and association with vitamin D, body composition, illness, injury, and skin infection prevalence.

3. Results

Twenty male wrestlers initially volunteered to participate in the study; nineteen wrestlers reported to baseline testing. All 19 initial participants completed baseline vitamin D testing (September), but body composition data could not be obtained on one athlete due to scheduling conflicts. Baseline characteristics for the 18 wrestlers for which body composition was available are summarized in Table 1.

Table 1. Baseline characteristics of 18 male wrestlers [1].

Age (years)	Height (cm)	Weight (kg)	BMI (kg/m^2)	Body Fat (%)
20.9 ± 2.0	171.8 ± 15.3	87.4 ± 18.6	27.3 ± 4.0	19.0 ± 7.0
19–23	162.6–193.0	62.0–121.7	22.6–35.4	12.5–37.7

[1] Data reported as mean ± SD with range listed beneath; Caucasian (n = 15); Spanish Italian (n = 1); Asian (n = 2). Abbreviations: BMI, body mass index.

By the winter data collection point (January), three participants discontinued participation for undisclosed reasons. Complete longitudinal data for vitamin D was therefore available on 16 wrestlers for winter and spring collection and logitudinal body composition and cytokine data were only available on 15 due to difficulty with a blood draw in the fall (n = 1) and scheduling conflicts with the DXA measurement (n = 1).

During the 2014–2015 academic season, the team as a whole participated in 5 tournaments, 15 dual meets, and six wrestlers competed in the NCAA Division I Wrestling Championships. Starters wrestled an average of 31 matches. Among the athletes who completed the study, there was representation from each of the ten weight classes and each class weight class included a conference dual starter with the exception of the 141 lb weight class in which only fall data was available.

3.1. Vitamin D Status

As shown in Figure 1 below, serum 25(OH)D changed across time ($p < 0.001$) and was highest in the fall and lowest in the winter. In the fall (n = 19), five athletes (26.3%) had sufficient vitamin D status whereas twelve (63.2%) and two (10.5%) presented with insufficient (20–32 ng/mL) and deficient status (<20 ng/mL), respectively. In the winter and spring (n = 16), one athlete (6%) had sufficient

status, and eleven (69%) and four (25%) had insufficient and deficient status, respectively. None of the athletes experienced optimal 25(OH)D status (>40 ng/mL) [5,11] at any time point across the season.

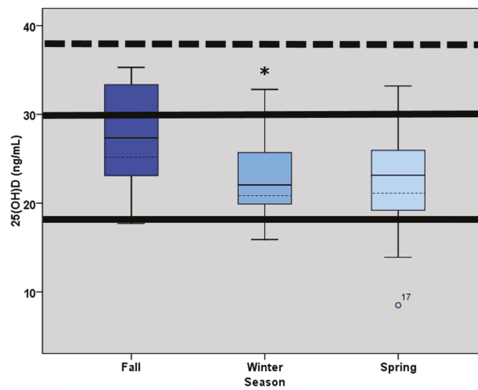

Figure 1. Box plots illustrating the distribution of 25(OH)D concentration (ng/mL) in the fall (n = 19), winter (n = 16), and spring (n = 16). Box extents indicate the 25th and 75th percentile, with the median indicated by a solid dark line and the mean indicated by a dashed line. Central vertical lines (whiskers) extend up to 1.5 interquartile ranges from the end of the box. A circle marks individual points outside of the whiskers indicating a value between 1.5 and 3.0 interquartile ranges of the box. 25(OH)D concentrations <20 ng/mL are considered deficient, concentrations between 20 and 32 ng/mL are considered insufficient (solid horizontal lines), and concentrations >40 ng/mL are considered optimal. * Significant decrease in 25(OH)D observed between fall and winter ($p < 0.001$), reported as mean ± SD.

3.2. Body Weight and Body Composition

Body weight changes over the course of the training season/academic year (September–May) ranged from −12.0 kg to +4.5 kg with an average season weight change of −3.4 ± 3.8 kg (n = 16, p = 0.003). Most of the weight loss, averaging 4.1 ± 2.4 kg (−1.0 kg to −9.5 kg), occurred between the fall and winter. This was followed by an average weight gain of 0.21 ± 2.4 kg (−6.0 ± 4.1 kg) per wrestler in between winter and spring. The trend for weight gain between winter and spring remained when omitting the four heavy weight wrestlers in the group (0.06 ± 0.12 kg), but was not apparent when accounting for the nine conference season starters (0.003 ± 0.171 kg). Among the entire group for which weekly weight changes were available (n = 19), mean week-to-week weight change (Monday to Monday) averaged −0.19 ± 0.10 kg (−0.37 kg to −0.06) over the course of the study. This minimal change in weekly weight became even smaller when accounting for season starters only (n = 10) and also when omitting heavyweights (n = 15). Despite minimal changes in weight between team weekly weigh-ins, the amount of weight lost each week to make competition weight, not including heavy weights (n = 15), averaged 4.6 ± 1.0 kg (6.3% ± 1.5%). Similar results were observed when accounting for starters. Average weekly weight change did not change across any time point (fall to winter, winter to spring, or fall to spring, $p > 0.05$).

As summarized in Table 2, body composition changed across the training season/academic year (n = 15, $p < 0.01$) despite the minimal week-to-week variability in body weight. Absolute total body mass, body mass index (BMI), fat mass, and body fat percentage decreased whereas lean body mass and bone mineral density (BMD) increased.

3.3. Cytokine Concentrations and Inter-Cytokine Relationship

As shown in Table 3, there was considerable variability in the concentrations of the various cytokines among individuals and across the academic year (n = 15). Concentrations of IL-6 and TNF-α

were numerically higher in the fall and spring and lowest in winter whereas concentrations of IL-10 demonstrated the opposite pattern. These slight alterations, however, were not different across time ($p > 0.05$).

Table 2. Body composition across the academic year [1].

Measurement	Fall	Winter	Spring	Sig. (p) [2]
Weight (kg)	90.6 ± 18.0	86.5 ± 17.0	86.7 ± 16.0	<0.001
BMI (kg/m^2)	27.6 ± 3.9	26.4 ± 3.6	26.7 ± 3.4	0.001
Lean Mass (kg)	69.0 ± 10.4	70.8 ± 9.8	70.5 ± 9.8	0.003
Fat Mass (kg)	17.6 ± 10.2	11.8 ± 9.4	12.1 ± 9.1	<0.001
Body Fat (%)	19.4 ± 7.1	13.2 ± 7.4	12.8 ± 5.0	<0.001
BMD (g/cm^2)	1.42 ± 0.1	1.45 ± 0.1	1.47 ± 0.1	0.001

[1] Data reported as mean ± SD; [2] Repeated measures ANOVA of body composition changes across time (n = 15) (statistic × time effect).

Table 3. Cytokine concentrations across the academic year [1].

Cytokine	Fall	Winter	Spring	Sig. (p) [2]
IL-6 (pg/dL)	110 ± 229	73 ± 144	120 ± 306	0.335
IL-10 (pg/dL)	254 ± 469	362 ± 938	246 ± 559	0.270
TNF-α (pg/dL)	1549 ± 2361	886 ± 1117	1293 ± 2197	0.287

[1] Data reported as mean ± SD. [2] Repeated measures ANOVA of body composition changes across time (n = 15) (statistic × time effect).

In addition, cytokine concentrations were highly inter-correlated at all time points (Pearson's). IL-6 was positively correlated with both IL-10 and TNF-α during the fall (n = 18), winter (n = 15), and spring (n = 15) (IL-10: $r = 0.924$, $r = 0.856$, and $r = 0.846$, $p < 0.001$, respectively; TNF-α: $r = 0.950$, $r = 0.837$, and $r = 0.945$, $p < 0.001$, respectively). IL-10 also positively correlated with TNF-α at all time points ($r = 0.897$, $r = 0.767$, and $r = 0.969$, $p < 0.001$, respectively).

3.4. Illness, Injury, and Skin Infection

Skin infection occurrence over the course of the season (September–May) averaged 1.8 ± 1.3 per participant with fifteen (79%) subjects experiencing at least one skin infection by the end of fall with four (11%) new infections being documented between winter and spring. A total of 35 skin infections were reported over the course of the season with a majority (n = 31, 89%) being reported between the fall and winter. Herpes (n = 19), ringworm (n = 4), and impetigo (n = 2) were the most common diagnoses between fall and winter while only herpes (n = 4) was reported between the winter and spring. The forehead was the most common skin infection site (n = 8) followed by the ear, back, arm/wrist, and lip being the second most frequent location (n = 4).

Eight subjects experienced a documented illness over the course of the study with one subject contracting two illnesses. Six of these were reported between the fall and winter and three were reported between winter and spring. The primary diagnosis was URTI (n = 8) which included cough, congestion, and sinus infection.

There were a total of nine injuries reported in seven different athletes over the year with an average of 0.5 ± 0.7 injuries per wrestler. Collectively, there were 50 total skin infections, illnesses, and injuries reported over the year averaging 2.6 ± 1.5 per wrestler (n = 19).

3.5. Vitamin D

3.5.1. Vitamin D Intake and Relation to Vitamin D Status

Intake of vitamin D from food alone or food plus supplements is summarized in Table 4. Athletes did not meet the recommended dietary allowance (RDA) for vitamin D intake from food alone (600 IU) at any time point during the year. When supplements were included, seven athletes met the RDA in the fall, five in the winter, and four in the spring. There were no significant changes in dietary vitamin D intake from food alone or food plus supplements across time ($n = 16$, $p = 0.25$ and $p = 0.707$, respectively).

Table 4. Vitamin D intake from food and supplements across the academic year [1].

Vitamin D Source	Fall	Winter	Spring
Dietary Intake	257 ± 212	211 ± 135	250 ± 191
Intake from Supplements	443 ± 996	370 ± 569	296 ± 758
Combined Vitamin D Intake	1549 ± 2361	886 ± 1117	1293 ± 2197

[1] Reported as International Units (IU), mean ± SD ($n = 16$).

Vitamin D intake from food alone or food plus supplements was not associated with serum 25(OH)D concentrations at any time point ($p \geq 0.05$). However, in the spring there was a positive association between both total vitamin D intake and vitamin D intake from supplements and vitamin D category (i.e., deficient, insufficient, etc.) such that those with higher overall vitamin D intake had higher categorical status ($n = 16$, $r = 0.563$, $p = 0.02$ and $r = 0.556$, $p = 0.02$, respectively).

3.5.2. Relation between Vitamin D Status and Body Composition

25(OH)D was negatively correlated with body fat percentage in the fall ($n = 18$, $r = -0.481$, $p = 0.043$), winter ($n = 16$, $r = -0.521$, $p = 0.038$), and spring ($n = 16$, $r = -0.565$, $p = 0.18$). 25(OH)D was correlated with fat mass in the spring only ($n = 16$, $r = -0.652$, $p = 0.005$) as shown in Figure 2, with a trend for a similar association in the winter ($n = 16$, $r = -0.446$, $p = 0.083$), but not the fall ($n = 18$, $r = -0.385$, $p = 0.115$). 25(OH)D, however, was not associated with total body mass, lean mass, or BMI at any time point ($p > 0.05$). There was an interaction with vitamin D category (sufficient, insufficient, and deficient) and total body mass ($p = 0.012$), fat mass ($p = 0.001$), and body fat percentage ($p = 0.001$) such that those with higher weight and adiposity had lower status. The change in vitamin D status from fall to winter and from winter to spring, however, did not appear to be associated with the change in total body mass or fat mass over these same time points ($p > 0.05$). However, average weekly weight fluctuations (Monday to Monday) from fall to spring positively correlated with spring 25(OH)D ($n = 16$, $r = 0.663$, $p = 0.005$,) such that those with larger average weekly weight changes had higher vitamin D status in the spring.

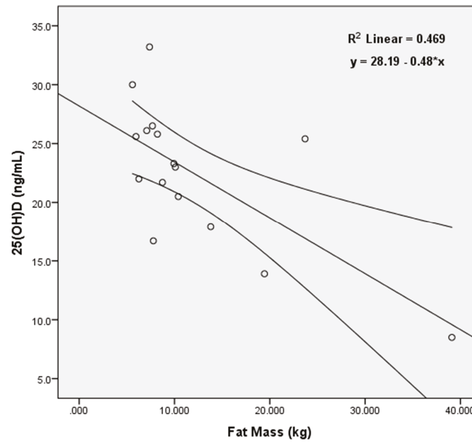

Figure 2. Linear regression model illustrating the association between 25(OH)D and fat mass (kg) in the spring (n = 16). Negative associations between these two variables were also observed in the fall and winter.

3.5.3. Relation between Vitamin D Status and Cytokines

Neither 25(OH)D or vitamin D category (sufficient, insufficient, and deficient) correlated with IL-6, IL-10, or TNF-α at any time point (n = 15, $p > 0.05$, Pearson's correlation).

3.5.4. Relation between Vitamin D Status and Illness, Injury, and Skin Infections

Neither 25(OH)D or vitamin D category (sufficient, insufficient, and deficient) was associated with number of illnesses or skin infections at any time point or over the course of the academic year (n = 15, $p > 0.05$), however, winter vitamin D category was positively associated with number of injuries incurred from fall to winter such that those with higher status experienced more injuries (r = 0.550, p = 0.020).

3.6. Cytokines

3.6.1. Relation between Body Composition and Cytokines

At no time point did body composition (total body mass, body fat percentage, fat mass, lean mass, or BMD) influence IL-6, IL-10, or TNF-α over time ($p > 0.05$). Fall and winter fat mass influenced the change in TNF-α over time that approached statistical significance (n = 15, $p = 0.62 \times$ time effect, $p = 0.73 \times$ time effect).

3.6.2. Relation between Weight Loss and Cytokines

Average weekly weight fluctuations over the course of the year (fall to spring) and cytokine concentrations were not correlated at any time point ($p > 0.05$). Significant correlations were found between weight change from fall to winter and winter cytokine concentrations (n = 15, Table 5).

Table 5. Association between cytokine concentrations in the winter and total body mass change between fall and winter (n = 15).

Spearman's Rank	*Correlation (r)*	Sig. (p)
IL-6	0.497	0.060
IL-10	0.561	0.029
TNF-α	0.540	0.038

3.6.3. Relation between Cytokine Concentrations and Prevalence of Illness, Injury, and Skin Infection

Cytokine concentrations were not associated with the prevalence of illness, injury, or skin infections at any time point during the study (n = 15, $p > 0.05$).

4. Discussion

This study evaluated the vitamin D status of male collegiate wrestlers over the course of an academic year and aimed to determine whether vitamin D status was influenced by vitamin D intake and body composition. We also aimed to determine whether vitamin D status influenced cytokine concentrations, injury, illness, and skin infection incidence. Furthermore, it was evaluated whether wrestlers who "cut" more weight over the course of the season, experienced greater season-induced changes in vitamin D status and/or cytokine concentrations. We found a high prevalence of vitamin D insufficiency and deficiency in wrestlers throughout the academic year, which ranged from 74% in the fall to 94% in the winter and spring, that was inversely associated with body fat percentage and was influenced by week-to-week weight fluctuations, but was not impacted by intake or overall weight loss throughout the season. An association between serum 25(OH)D concentration and risk of injury, infection or illness was not observed.

Despite an initially high prevalence of vitamin D insufficiency and deficiency in the fall, the status of college wrestlers declined significantly across the academic year as has been previously shown in other athlete groups [11,25–30]. The majority of the decline occurred between the fall (September) and winter (January) with the incidence of insufficiency/deficiency reaching 94% in the winter and spring. The overall prevalence of suboptimal status was higher than previously reported in our laboratory in college athletes from mixed sports [11,24] and those of other indoor athletes [26,27] and sportsmen training near the equator where suspected sun avoidance was the culture [31]. Although it was not surprising that wrestlers had low vitamin D status in the winter and spring due to their exclusive indoor training regimen, it was somewhat surprising that status was so low in the fall when wrestlers engaged in close to eight weeks of outdoor training typically between 2:00 and 5:00 p.m. in mostly sunny conditions at an elevation of between ~7200 and 8400 feet [25]. The higher than expected prevalence of insufficiency and deficiency may be a combination of late afternoon training, which misses peak hours of 10:00 a.m.–2:00 p.m., clothing worn, or the higher body fat percentage of some of the athletes [16] when weekly weigh-ins are not required. In agreement with previous studies in both athletes and non-athletes [16,30,32–34], body adiposity was negatively associated with serum 25(OH)D concentration across the academic year. Although the specific mechanism for this association is not fully understood, the lipophilic properties of vitamin D are thought to allow sequestration in adipose tissue, which thereby decreases circulating 25(OH)D concentrations [30,35].

The overall low vitamin D intake may have also contributed to low status. Vitamin D intake was, on average, less than 50% of the RDA across the season, which is consistent with research in other athletic populations [11,25,26,36]. Vitamin D supplementation was also low. For instance, depending on the time of the year, only two to three wrestlers reported taking a vitamin D supplement and only five to six reported taking a multi-vitamin. In agreement with previous studies [11,32], vitamin D intake from food or supplements was not directly associated with vitamin D status. Ironically, in the current study the wrestler with the lowest serum 25(OH)D concentration had the highest body mass, the third highest body fat percentage, and reported taking a vitamin D supplement during the entire

academic year. He also experienced a significant decline in body fat percentage (37.7% in the fall to 19.6% in the spring). This suggests that the supplemental dose taken (not reported) was not sufficient to counteract the negative influence of adiposity on status across the season despite a significant reduction in body adiposity.

Unique to this study was our evaluation of whether vitamin D status impacted cytokine concentrations over the course of the year. Our initial hypothesis was that athletes with lower vitamin D status would have higher pro-inflammatory cytokines and lower anti-inflammatory cytokines than those with higher status. Our lack of an association between serum 25(OH)D and cytokine concentrations during the season, however, may be explained by the overall low vitamin D status of our wrestlers and/or the high variability in cytokine concentrations throughout the year. Although all blood cytokine concentrations were obtained after at least 12 h of physical inactivity, approximately eight weeks (August–September) of conditioning (6 days/week) and strength training (3 days/week) occurred before the initial data collection in September. Thus, preseason training may have elevated cytokines in some or all athletes. It is not well established how a single bout of exercise or regular training influences cytokine concentrations, or how long athletes should refrain from exercise to reveal cytokine samples reflective of overall health. For example, some research has shown that after a single bout of intense physical activity, cytokine concentrations return to baseline within 2–3 h after exercise terminates [9,37] while others suggest that cytokines remain elevated even 24 h following exercise [38,39].

In the present study, IL-6 and TNF-α concentrations decreased between fall and winter. This was followed by an increase back toward the fall baseline between winter and spring. IL-10 concentration, in contrast, increased between the fall and winter also returning toward baseline in the spring. The decrease in TNF-α concentration between the fall and winter was somewhat unexpected due to intense practices and weight training sessions 5–6 days per week, which would be expected to induce a low level inflammatory response. Perhaps, TNF-α was not elevated due to a concurrent rise in IL-10 concentrations. IL-10 is a potent anti-inflammatory cytokine that has been shown to assist in the down-regulation of TNF-α during exercise [37,40]. In agreement with our findings, previous research has also shown a lack of correlation between 25(OH)D and TNF-α [32,41–45]. Our lack of a relationship between vitamin D status and TNF-α concentration, however, is contradictory to Willis et al. [24], and others who have found a significant inverse relationship between 25(OH)D and TNF-α concentrations in both humans and mice [45–48].

In the current study, the majority of skin infections were reported between the fall and winter (n = 31) as compared to between winter to spring (n = 4). This was somewhat expected, as wrestlers transition from summer conditioning and strength training to training in the wrestling room where skin-to-skin and skin-to-mat contact are more frequent. This time period also coincides with the suspected reduction in cutaneous vitamin D synthesis (from limited sun exposure and reduced synthesis capacity) and frequent dietary restriction as wrestlers work to maintain competition weight on a week-to-week basis. As previously noted, the majority of the decrease in serum 25(OH)D concentration occurred between fall and winter (28.2 $\pm$ 5.2 vs. 22.8 $\pm$ 4.6 ng/mL), however, vitamin D intake remained unchanged despite the suspected intentional energy restriction, at least in some wrestlers. We had hypothesized, however, that the increased skin infection risk would be associated with low serum 25(OH)D. The hormonally active form of vitamin D, $1,25(OH)_2D_3$, has been shown to up-regulate expression of anti-microbial peptides (AMP's) and assist in the defense against bacterial infection [49]. Our lack of association between vitamin D status and frequency of both skin and other documented illnesses, nevertheless, may be due to the overall high frequency of vitamin D insufficiency and deficiency. This precluded maintenance of 25(OH)D concentrations in a range not high enough to experience a beneficial effect. For example, when Halliday et al. evaluated the relationship between vitamin D status and illness in collegiate athletes from our same university, athletes with higher 25(OH)D concentration in the winter and spring were found to experience fewer illnesses over the course of the year [11]. Reduction in illness frequency, however, was not apparent until serum 25(OH)D

concentrations approached approximately 40 ng/mL [11]. In the current study, only one wrestler maintained vitamin D status in the sufficient category (>32 ng/mL) in both winter and spring and none maintained 25(OH)D concentration greater than 40 ng/mL. Future research should include a vitamin D supplementation trial that elevates concentrations of 25(OH)D above 40 ng/mL to allow for a better analysis of the impact of maintaining optimal vitamin D status on inflammation, illness, and skin infection in wrestlers.

The present study did not reveal a consistent relationship between vitamin D status and number of injuries sustained. This may be partially explained by our low injury rate, injury underreporting, or small sample size. In the present study, our injury frequency averaged approximately 0.5 injuries per wrestler which was lower than previous reports in male ballet dancers (1.9 injuries/dancer) [50], Taiwanese elite wrestlers (4.2 injuries/wrestler) [51], and high school wrestlers (5.2 injuries/wrestler) [52]. With the identification of vitamin D receptors in skeletal muscle cells [53], it is not surprising that several previous studies have found associations between higher vitamin D status and fewer incidences of injuries, including in professional American football athletes [54,55]. Furthermore, vitamin D supplementation in ballet dancers has been shown to reduce injuries over a 4-month period when compared to no supplementation [7]. Other research has indicated that while maintaining higher vitamin D concentrations may not prevent injury, it may assist in expediting recovery [56]. One unexpected result of the current study was the positive correlation between vitamin D status in the winter and the number of injuries that occurred between the fall and winter. It is likely that this association is coincidental due to the small number of injuries and small sample size. Overuse, over-training, and/or poor wrestling technique may also confound our findings. Unfortunately, duration of injury or injury rehabilitation response was not evaluated in the present study.

Collectively, the reported total number of skin infections, illnesses, and injuries averaged 2.7 ± 1.5 per wrestler ($n = 20$), however, actual rates may be higher due to underreporting by the athlete to avoid restrictions from practice and/or competition. The five injuries that occurred in the four wrestlers between fall and winter included a concussion, wrist fracture, meniscus tear, foot sprain, and a shoulder pain complaint due to surgery that occurred at the beginning of the semester.

We found that fat mass at all three time points (fall, winter, and spring) influenced the change in TNF-α across time. This finding is in agreement with previous reports indicating a positive association between adiposity and inflammation [36]. This may warrant special attention to those athletes with higher adiposity, as they may be more prone to injury and illness. Interestingly, a non-significant inverse relationship between all winter cytokine concentrations and the total number of illnesses, injuries, and skin infections was observed, implying that between September and January, a period when training is at its peak, lower cytokine concentrations were associated with increased risk for illness, skin infection or injury.

In the current study it is important to note that over the course of the season wrestlers lost an average of 3.5 kg (-12.0 to $+4.5$ kg), which is lower than the 7.9 ± 0.8 kg loss reported by Melby et al. [57]. On average, wrestlers started the training week (Monday) 4.6 ± 1.0 kg ($6.3\% \pm 1.5\%$) over their weight class suggesting an approximate 5 kg weight loss each week (Monday–Friday) to "make weight" for competition. The weight loss observed in this study is on the lower end of the previously reported weekly loss of 5.0–9.1 kg reported by Steen and Brownell [19], but higher than the 2.3 and 3.4 kg deficits observed by Lakin, Steen, and Oppliger [20] and Lingor and Olson [21], respectively. Furthermore, average weekly weight loss, Monday to Monday, averaged <0.5 kg which is significantly lower than the suggested weight loss between Monday to Friday and lower than any of the aforementioned studies [19–21]. No significant differences in average weekly weight loss between starters and non-starters were observed. This may be due to non-starters competing in non-conference dual meets on the weekends requiring them to engage in similar weight management behaviors as starters. Culturally, coaches also implemented a "maximum weight over" policy that prohibited wrestlers from participating in normal practice activities if they reported to Monday training more

than 4.6 kg over their weight class. The positive association between average weekly weight change (Monday to Monday) over the course of the season and spring 25(OH)D concentration and status implies that those who had larger weekly weight fluctuations tended to have higher vitamin D by the spring, which is in contrast to our hypothesis. While unexpected, this observation may reflect a release of vitamin D from adipose tissue by those wrestlers who lost more weight each week.

Limitations

Although our study captured a unique group of athletes potentially at increased risk for poor vitamin D status, it has several limitations. This includes the small sample size, limited racial differences, and the limited variability in 25(OH)D among individual wrestlers at all-time points. For example, a majority of wrestlers in fall, winter, and spring had serum 25 (OH)D concentrations in the deficient and insufficient range with only a few athletes ($n = 5$ in the fall and $n = 1$ in the winter) with concentrations in the sufficient range and none in the optimal range. This precludes comparisons between those with deficient/insufficient status and those sufficient or optimal status, and also limits statistical power. In addition, participants in the current study were closely monitored by a registered dietitian (J.N.B.) which may have positively influenced dietary habits (resulting in better fueling choices, healthier weight loss practices, and lowered post-exercise cytokine responses). The "maximum weight over" policy established by the sport coaches to prevent wrestlers from reporting to Monday practice more than 4.6 kg over their weight class, may have reduced week-to-week weight fluctuations. The low number of skin infections, illnesses, and injuries reported in the current study between the winter and spring may be the result of under-reporting, under-recording, or season-to-season variability of which made detection of relations with vitamin D status difficult. In addition, data collection, particularly for cytokine concentrations, began after approximately eight weeks of fall conditioning (August-September) which may have altered the true picture of how vitamin D status influences such concentrations during training and competition, however, they are a reality for the in-season college athletes. Finally, vitamin D binding protein (VDBP) and gene polymorphisms were not addressed in this study which may have influenced 25(OH)D concentrations [58,59].

5. Conclusions

This study is the first, to our knowledge, to directly analyze the relationship between vitamin D status' and weight cycling's impact on inflammation, skin infection, illness, and injury in wrestlers. Overall, there was a lack of association between vitamin D and prevalence of illness, injury, and skin infection which was in partial contrast to our hypothesis and supports the need for further research in wrestlers. Future research should attempt to analyze a larger sample size, include a supplementation group, and also include wrestlers from varying latitudes. Although we were not able to shed light on the ability of vitamin D to influence cytokines or reduce risk of illness, injury, or infection, our study revealed that despite the stereotypical extreme weight making practices of wrestlers, this group experienced minimal weekly weight fluctuations to reach their goal weight and this likely contributed to their ability to maintain or gain lean body mass while decreasing body fat over the course of the year. Although most of the wrestlers did not utilize vitamin D supplements, exploring the potential benefits of vitamin D supplementation for reducing frequency of illness, injury, and skin infections should be explored in future research especially in a group that appears to have a high prevalence of insufficiency and deficiency.

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