Micronutrient Supplementation does not Change Complement System Response to Heavy Training

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ABSTRACT

We aimed to examine the micronutrient supplementation effect on complement system activity after heavy training. 24 male firefighters were randomly divided into supplemented and placebo groups, and tested for immunology-related parameters using venous blood samples in the fasting state pre- and post-5 weeks of nutritional supplementation. C3 and C4 complement components were determined in a nephelometer from immune complexes formed through specific human antisera and total haemolytic complement activity (CH100) was determined by enzyme immunoassay. Differences between pre- and post-supplementation were observed only for CH100 on placebo group (p = 0.004; mean diff − 26.92; 95 %CI − 43.58 to − 10.25) and no interaction, treatment or time effects were observed for C3 and C4. Although interaction accounted for 8.8 % of the total variance in CH100 (with time effect pre- vs post-accounting for 19.5 % of the total variance), the treatment effect (supplemented vs placebo) was not significant. The absence of effects on the complement system response to supplementation during heavy training could be justified by the fact that: (i) nutritional supplements do not improve humoral innate immunity in well-fed subjects; (ii) selected supplements unlikely improve the innate immune system in situations of adequate nutritional status; and/or (iii) selected doses of supplementation were not sufficient to elicit immune changes.

Introduction

The complement system is an innate immunity key component consisting of proteolytic cascade paths activated by pathogenic microorganisms, immune complexes and auto activation of structurally unstable C3. Corresponding to the lectin classical and alternative pathways, they lead to formation of a lytic membrane attack complex [23]. Complement plays an important role in inflammation, foreign materials opsonisation, phagocytosis facilitation and direct cytotoxic reactions, working as an antibody-dependent effecter to eliminate pathogens [43]. It regulates several adaptive immune responses and is conditioned by sleep and circadian rhythms, environmental temperature and humidity, ethnicity, physical activity levels, disease, specific nutritional status and ano-

rexia nervosa [23, 28, 34]. C3 and C4 complement components are not sensitive to acute psychological stress [35], but although depressive disorders do not affect C3, they might increase C4 serum levels [3].

It has been suggested that exigent physical conditioning elicit changes in the peripheral blood cellular and humoral components of the immune system [7]. This change is related to inflammatory and oxidative stress markers [20] with prolonged exercise and heavy training loads associated with depressed immune function [14]. In fact, well-trained individuals have lower C3 and C4 resting levels [27] and are prone to upper respiratory diseases [13]. Furthermore, nutritional status can directly affect well-trained subjects' immune response to heavy training, because high carbohy-

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drate intake during prolonged exercise limits exercise-induced immune depression [14] and inadequate nutrition negatively influences immunecompetence after heavy exertion [15]. In addition, cellular immunity responds better than humoral immunity to nutritional supplementation, as glutamine supplementation improves: (i) cellular (but not humoral) immunity functions in severely depressed immune system subjects and (ii) cellular immunity (like CD4/CD8 ratio), although IgG, IgM, C3 and C4 plasma concentrations did not change in severe burn patients [29].

Dietary protein and specific micronutrients deficiencies have been associated with immune dysfunction, but benefits regarding high doses of anti-oxidant intake are not sufficiently studied. This is very relevant once antioxidant vitamins and trace elements modulate immune cell function through regulation of redox-sensitive transcription factors [44], although this supplement effect on immune humoral function is not well investigated. Since the complement system is a central mediator of inflammation [43], its improvement might elicit some immune surveillance against exercise-induced inflammatory focus. We aimed to verify if supplementation with antioxidant vitamins, minerals and trace elements can alter immune humoral function and total complement activity after a period of heavy physical exertion. It was hypothesised that supplementation induces complement system benefits post-heavy physical training.

Material and Methods

Sample

24 male firefighters volunteered to participate and were randomly divided into supplemented and placebo groups (concealed allocation was implemented). The inclusion criteria were that subjects were professional firefighters; healthy (assessed through medical tests); with no muscular, bone or articular pathologies and visual or hearing deficits; and with a positive classification in physical conditioning tests. Subjects with any incapacitating physical or organic pathology were excluded. There were no differences between groups regarding age, anthropometrical and physical conditioning characteristics (▶ **Table 1**), and no dropouts occurred during the study. Experimental procedures were conducted in accordance with Helsinki Declaration and ethical principles for medical research involving human subjects [16].

Testing protocol

The current study was randomized, double-blinded and placebo-controlled with supplemented and placebo groups receiving, over 35 consecutive days, a proprietary supplement (Ever-Fit Plus, Prisfar [R] with 15 mg of beta-carotene, 200 mg of vitamin C, 136 mg of vitamin E, 200 µg of selenium, 15 mg of zinc and 100 mg of magnesium) and a placebo powder (maltodextrin with artificial flavour and colour), respectively. The training period included 5 microcycles of 5 training units (including 30 min of military drills plus 90 min of technical skills with and without fire protective clothing) and 2 resting days. This and the contents of the weekly physical conditioning program are displayed in ▶ **Fig. 1**. Participants avoided physical exertion over the weekends during the study period. Anthropometrical evaluation included stature; body mass; tricipi-
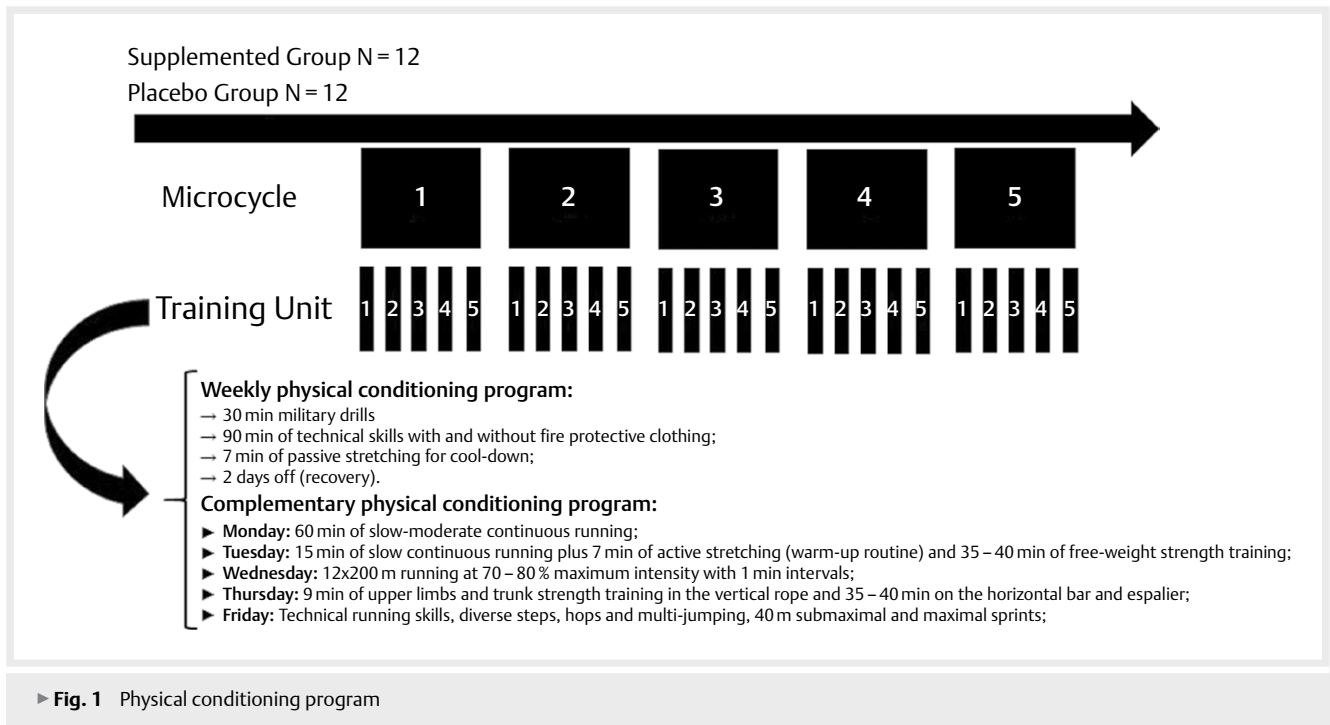
▶ **Table 1** Means plus SD values of age, anthropometrical and physical conditioning characteristics of the participants.

Variables	Supplemented group (n = 12)	Placebo group (n = 12)
Age (years)	22.1 ± 1.9	23.9 ± 0.3
Height (cm)	174.5 ± 3.8	174.3 ± 3.5
Body mass (kg)	68.9 ± 7.4	69.3 ± 12.3
Fat mass (% body mass)	9.6 ± 2.0	10.2 ± 2.0
Bench press with 50 kg (reps)	12.0 ± 7.1	13.7 ± 5.7
Chin-ups (reps)	15.8 ± 0.7	15.7 ± 2.1
Sprint 50 m (s)	7.00 ± 0.0	6.97 ± 0.05
Cooper test (m)	3 007 ± 127	3 120 ± 106
Relative peak power output (watt/kg) *	10.5 ± 0.5	10.8 ± 0.1
Relative mean power output (watt/kg) *	7.8 ± 0.5	8.1 ± 0.3
Relative minimum power output (watt/kg) *	5.7 ± 0.8	5.8 ± 0.6
Fatigue index (%) *	45.9 ± 6.4	45.2 ± 6.8
Squat jump (cm)	37.3 ± 5.7	37.8 ± 3.5
Countermovement jump (cm)	38.3 ± 6.2	39.4 ± 5.8
* Determined through the Wingate test		

tal, bicipital, sub-scapular and supra-iliac skinfold thickness; and fat mass [39].

All participants received the same physical conditioning and professional skills program during 3 months prior to experimentation, and presented a remarkable similarity in physical fitness in-between groups at the beginning of the study (▶ **Table 1**). Dietary intake was assessed on 2 weekdays and one weekend day record (one week before the intervention) using a photo album with 134 images containing average raw/cooked food portions. Mean daily food intake was converted to nutrients using Food Processor Plus [1] and no differences between groups regarding pre-supplementation values were observed (▶ **Table 2**). As groups had similar nutritional intake before intervention and firefighters had the same meals during the intervention, they were not tested again for these variables.

Venous blood was drawn from the antecubital vein in a fasting state after 2 resting days in pre- (3 months after the start of training activities) and post-5 weeks of supplementation. Complement components concentration in serum was determined using specific antisera to human C3 and C4 (Codes OSAP and OSAO), with the immune complexes formed measured in a nephelometer (Dade Behring Marburg GmbH, Newark, USA). C3 and C4 were calculated by comparison with known concentration standards. Total haemolytic complement activity (CH100) was determined in human serum by enzyme immunoassay in conjunction with the DiaSorin CAE Kit (Stillwater, Minnesota 55082-0285, USA). Activation level was expressed in complement activation by enzyme immunoassay units. In an attempt to avoid data analysis bias due to analytical variability, all blood samples were analysed in a single laboratory following the same analytical procedures. Main reference values in the literature are 86–184 and 90–180 mg/dl for C3, 20–58 and 10–40 mg/dl for C4, and 63–145 U/ml for CH100 [8, 19].

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▶ **Fig. 1** Physical conditioning program

▶ **Table 2** Means plus SD values regarding nutritional intake before supplementation.

Indicators	Supplemented group (n = 12)	Placebo group (n = 12)
Energy intake (kcal)	3 050 ± 600	2 985 ± 580
Carbohydrate (%)	51.2 ± 5.8	50.8 ± 3.1
Protein (%)	18.4 ± 2.5	19.2 ± 2.1
Fat (%)	30.2 ± 3.3	30.8 ± 2.8
Vitamin C (mg)	147.2 ± 58.2	152.8 ± 47.3
Vitamin E (mg)	7.9 ± 2.3	8.3 ± 2.8
Beta-carotene (μg)	165.1 ± 4.3	158.3 ± 7.2
Magnesium (mg)	431.4 ± 76.8	411.9 ± 82.5
Selenium (μg)	163.3 ± 22.7	161.9 ± 54.2
Zinc (mg)	16.9 ± 5.7	17.1 ± 4.2

Statistical analysis

A sample size of 24 subjects was deemed adequate (software G * Power 3.1.9.2© Heinrich-Heine-Universität Düsseldorf, Germany), assuming 85 % of statistical power and 0.05 α error probability. Data were first tested for distribution normality and variance homogeneity. A paired measures t-test was used to compare C3, C4 and CH100 values on pre- and post-supplementation conditions for each group. Then, the effects of treatment (supplemented vs placebo) and time (pre- vs post-supplementation) were assessed for each variable using a 2-way ANOVA. When a significant effect was found, the Bonferroni post hoc procedure was performed to localize the difference. Statistical Package for Social Sciences 19.0 was used, with results presented as mean plus standard deviation and statistical significance set at $p < 0.05$.
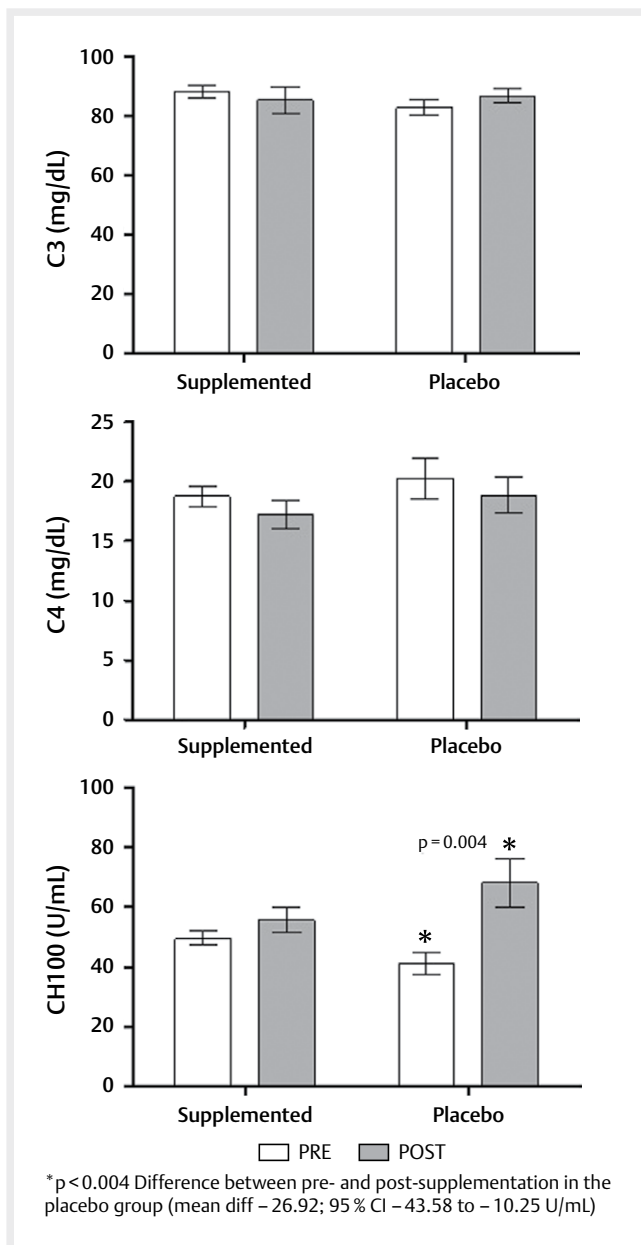
Results

Regarding micronutrient supplementation (concurrent with heavy physical training), differences for pre- and post-test were observed only for CH100 in the placebo group (p = 0.004; mean diff − 26.92; 95 %CI − 43.58 to − 10.25; cf. ▶ **Fig. 2**).

ANOVA showed no interaction, treatment or time effect for C3 and C4 (▶ **Table 3**). Although interaction accounted for 8.8 % of the total variance in CH100 ($F_{(1,44)} = 4.249$, p = 0.045, partial $\eta^2 = 0.088$; observed power = 0.522), with time effect accounting for 19.5 % of the total variance in CH100 ($F_{(1,44)} = 10.662$, p = 0.0021, partial $\eta^2 = 0.195$; observed power 0.891), the treatment effect was not significant ($F_{(1,44)} = 0.144$, p = 0.670, partial $\eta^2 = 0.04$; observed power = 0.522).

Discussion

Literature relating to physical training, nutrition and immune humoral system is very scarce, with the current study giving new insights into the influence of several nutrients in humoral immune response in very exhaustive training. The hard physical loads that typical elite athletes (and firefighters) experience induce necrotic material proliferation that can be cleared by complement system activation [23]. This allows opsonisation of damaged tissue prior to its ingestion by phagocytic leukocytes [38, 43]. Complement pathway activation seems to be independent of exercise type, since aerobic and anaerobic exercises induce similar changes (C3 and C4 serum levels decrease after both a 30 s anaerobic test and 30 min treadmill running [14, 18]). However, some data are contradictory, because C3 and C4 rose after maximal cycling [10] but no alterations were detected immediately after long-lasting exercise [37]. In addition, C3 and C4 values rose during and immediately after 2.5 h of running (with C4 continuing to rise some hours after the

$*p < 0.004$ Difference between pre- and post-supplementation in the placebo group (mean diff – 26.92; 95 % CI – 43.58 to – 10.25 U/mL)

▶ **Fig. 2** Micronutrient supplementation in both groups

quickly to basal levels when exercising at moderate intensity [33]. It was observed that runners had lower basal C3 and haemolytic activity values than non-exercising controls [14, 40], with literature differences justified by diverse subjects' physical condition or methodological discrepancies.

In general, exhaustive physical loads induce an acute increase of several complement system components [4, 7, 43], with systematic heavy physical training depressing their basal values [14, 44]. This was observed in the current study's C3, C4 and CH100 data, evidencing a chronic adaptation to high-intensity training loads. Our subjects' average basal serum C3 levels were close to the lowest reference values [8, 19] and similar to those found in professional cyclists [36]. The current study's serum C4 levels were lower than some clinical references [19] but within the normal range [8, 36]. Although not observed in our experiment (cf. ▶ **Fig. 2**), basal C3 and C4 levels tend to be lower in well-trained subjects [27, 44]. However, differences between sports should be considered, because no modification of C3 and C4 serum levels was observed during a volleyball season [6] despite decreased basal C3 values through aerobic cardiovascular training [27, 30].

In the current study, total complement activity (CH100) at pre-supplementation was lower than laboratory references. This provides insight into the integrity level of the entire classical complement pathway, which is usually elevated in inflammation and infection situations, and decreases with fasting and malnutrition [24]. Although haemolytic activity decreases after short-term aerobic exercise [40], analysis of CH100 alterations induced by heavy physical training is required. From the current data, we speculate that low basal CH100 values, in the absence of any known disease or nutritional deficiency, evidence haemolytic activity attenuation subsequent to systematic training. Moreover, CH100 increased in the placebo group, but no differences between groups were observed both at pre- and post-intervention. However, an increasing tendency after the intervention period was seen for this variable in both groups, suggesting that physical training continuity accentuated haemolytic activity independently of the supplementation.

Complement system enhancement through nutritional interventions is not new, with reduced nutritional intake promoting a decrease in serum C3 and C4 levels [15, 20], whereas diets inducing elevated serum low-density lipoprotein cholesterol increased C3 [21]. It is also known that vitamin D deficits are inversely related to C3 serum concentration [31], whereas exercise-induced dehydration increases C3 and C4 serum levels [5]. Dehydration was not taken into consideration in the current study because blood samples were drawn after a 48 h post-exercise recovery period.

It is accepted that complement system response can be improved by supplementation (at least in situations of nutritional deficits), with cyanocobalamin treatment improving the immune status of vitamin-B12-deficient patients by increasing C3 and C4 levels [11]. However, in well-fed subjects, nutritional supplementation effects are equivocal, because ingestion of mangosteen, multivitamins and essential minerals increased C3 and C4 serum concentration [42] but arginine supplementation did not modify their values [25]. It is also known that histidine-rich glycoprotein binds strongly to several complement proteins, contributing to the maintenance of normal immune function, inhibiting the formation of

end of the exercise) [9, 43], but serum complement decreased after 1–2 h of recovery in some individuals [26].

C3 and C4 serum concentrations rapidly return to pre-exercise levels after short-term maximal exercise [10, 27], but stay slightly lower after an ultramarathon [37], evidencing an exercise mode-related behaviour. These variables are also exercise time-dependent, as long-lasting physical activities induce lower basal C3 levels compared to intermittent exercise, and intermittent sports lead to higher C4 values than running [3]. Serum opsonic activity and C3 and C4 concentrations did not change immediately after a 100 km ultramarathon, but recovery can increase their activation towards the muscle debris and other altered molecules' phagocytosis [32]. Heavy exertion elicits strong complement activation, returning

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► **Table 3** Values (mean ± SD and 95 %CI) of C3, C4 and CH100 before and after the training period for supplemented and placebo group.

	Pre				Post			
	Supplemented	95 % CI	Placebo	95 % CI	Supplemented	95 % CI	Placebo	95 % CI
C3 (mg/dL)	88.2 ± 7.3	82.1 to 94.2	82.9 ± 9.0	76.8 to 89.0	85.4 ± 15.0	79.3 to 91.5	86.8 ± 8.8	80.7 to 92.9
C4 (mg/dL)	18.8 ± 3.1	16.1 to 21.4	20.3 ± 5.7	17.5 to 22.9	17.3 ± 4.1	14.5 to 19.9	18.8 ± 5.2	16.1 to 21.5
CH100 (U/mL)	49.6 ± 8.1	39.4 to 59.8	41.3 ± 12.8	31.1 to 51.5	55.7 ± 14.3	45.5 to 65.8	68.3 ± 28.1	58.1 to 78.4

insoluble immune complexes (enhancing complement activation) and promoting the faster clearance of necrotic materials [23]. Furthermore, supplementation with a substance isolated from Greenland shark liver increased C1q, C3 and C4 serum levels, improving innate immunity [17].

In the current study, vitamins C and E, β-carotene, selenium, zinc and magnesium did not enhance C3, C4 and CH100, meaning that humoral immunity responds differently than cellular immunity to supplementation even in situations of great immune stress. Similarly, cellular immunity in severely burned patients was enhanced by glutamine supplementation, whereas humoral immunity did not change [1]. Some reasons might justify the current study data: (i) in well-fed subjects, nutritional supplements do not improve humoral innate immunity; (ii) the selected supplements unlikely improve innate immune system in situations of adequate nutritional status; and/or (iii) the selected micronutrient doses were not sufficient to elicit immune changes.

No differences between groups were observed both at pre- and post-intervention, except for CH100 that increased in the placebo group. Although time effect accounted for 19.5 % of its total variance, treatment effect was not significant, suggesting an accentuated haemolytic activity with physical training, independent of supplementation. Although it was not possible to verify the same behaviour for all studied variables, the current study's low basal CH100 values can be the outcome of systematic heavy physical training loads that professional firefighters are accustomed to (worldwide they are typically engaged in this type of heavy physical conditioning). Therefore, these low values are not related to any nutritional inadequacy, because they did not change after micronutrient supplementation (neither did body or fat mass values). Therefore, albeit some studies point out complement system potentiation after supplementation, data should be carefully analysed, because even in situations of increased susceptibility to various infections, micronutrient supplementation can enhance immune response without complement system changes. In type 2 diabetes mellitus patients, vitamins D, E, B1, B2, B6 and C, folic acid, calcium, iron, zinc and selenium supplementation improved immune response to common infections without altering basal C3 and C4 values [17]. The current study participants showed an adequate intake (within RDI) of many micronutrients that are crucial for several components of innate immunity [22], partially justifying the observed ineffectiveness of supplementation.

Lastly, it is important to highlight that different supplementation types probably induce diversified results, necessitating future studies that apply different methodologies. In fact, intensive systematic running induced a C3 decrease delay, restored after administration of ectisten containing tincture of leuzea and leveton [2], and phytoecdysteroids seem to accelerate recovery of immune system affected by exhausting physical work [41]. Nevertheless,

large doses of vitamin C did not change C3, C4 and CH100 in actively immunized animals [12]. Therefore, the stability of low C3, C4 and CH100 levels in our subjects, even after heavy physical training with everyday supplementation, can be explained by the attenuation of the inflammatory response to the physical loads, evidencing a good training adaptation.

We do not consider relevant the differences in CH100 between groups after supplementation due to the low values obtained. In fact, the higher value obtained in the placebo group was close to the inferior limit, evidencing that this indicator was depressed in both groups in the beginning of the intervention and that supplementation did not alter the situation. We might conclude that 5 weeks of multivitamin and multi-mineral supplementation during heavy training in well-trained and well-fed individuals did not elicit any relevant changes in serum concentration of humoral immunity biomarkers. Future research should further investigate the underlying mechanisms and test the immunity responses to this kind of supplementation in subjects who are not so heavily trained.

Acknowledgements

Conflict of Interest

The authors declare that they have no conflict of interest.

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