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Spirulina supplementation prevents exercise-induced lipid peroxidation, inflammation and skeletal muscle damage in elite rugby players

Running title: Effects of spirulina in athletes

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Key points:

- This is the first study to investigate the effects of spirulina supplementation on pro/antioxidant status, inflammation, and muscle damage markers in response to exhaustive exercise in elite team-sport athletes.
- Spirulina supplementation potentially prevents exercise-induced lipid peroxidation, inflammation, skeletal muscle damage and may accelerate the recovery of these markers.
- Spirulina supplementation could be recommended for athletes who perform a high training load in order to reduce the magnitude of oxidative stress, inflammation, and muscle damage which could help to limit performance losses and accelerate recovery after training/competitions throughout the season.

Abstract

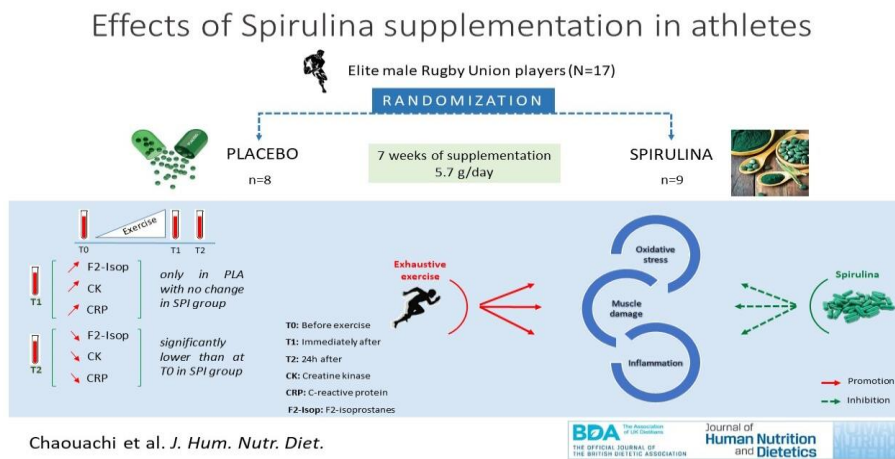
Objective: This study aimed to examine the effects of spirulina supplementation on pro/antioxidant status, inflammation, and skeletal muscle damage markers immediately and 24h after exhaustive exercise in elite rugby players.

Methods: Seventeen elite male Rugby Union players were randomly assigned to a Spirulina (SPI: n=9), or a placebo group (PLA: n=8) in a double-blind design. Subjects were supplemented with Spirulina platensis (5.7 g/d) or placebo (isoproteic and caloric) for 7 weeks. At baseline (W0) and after seven weeks of supplementation (W7), blood samples were obtained before (T0), immediately after (T1), and 24h after (T2) exhaustive exercise. The Yoyo Intermittent Recovery Test Level 2 was used as an exhaustive exercise to induce oxidative stress (OS), inflammation, and skeletal muscle damage. The studied parameters included Pro/antioxidant status markers (SOD, GPX, GSH/GSSG ratio, ox-LDL, and F2-Isop), inflammation markers (MPO and CRP), and skeletal muscle damage markers (LDH and CK).

Results: Our results showed that F2-Isop, CRP, and CK levels significantly increased at T1 only in PLA group ($p < 0.05$, $p < 0.05$, and $p < 0.001$ respectively) with no change in SPI group which reflects the effect of spirulina to prevent lipid peroxidation, inflammation, and skeletal muscle damage induced by exhaustive exercise. Moreover, spirulina supplementation accelerated the return to baseline values given that F2-Isop, CRP, and CK levels at T2 were significantly lower than at T0 in SPI group ($p < 0.05$, $p < 0.01$, and $p < 0.001$ respectively).

Conclusion: Based on the markers used in this study, our results report that spirulina supplementation potentially prevents exercise-induced lipid peroxidation, inflammation, skeletal muscle damage and may accelerate the recovery of some of these markers. Based on our findings, we recommend spirulina supplementation especially for athletes who do not achieve the recommended antioxidant dietary intake and who perform a high training load in order to reduce the magnitude of OS, inflammation, and skeletal muscle damage which could help to reduce performance losses and accelerate recovery after training/competitions throughout the season.

Graphical Abstract



Key words: Arthrospira; oxidative stress; high-level athletes; dietary supplement; antioxidant.

List of abbreviations

CK: Creatine kinase

COX-2: Cyclooxygenase-2

CRP: C-reactive protein

F2-Isop: F2-isoprostanes

GPX: Glutathione peroxidase

GSH: Reduced glutathione

GSSG: Glutathione disulfide

iNOS: Inducible nitric oxide synthase

LDH: Lactate dehydrogenase

MDA: Malondialdehyde

MPO: Myeloperoxidase

OS: Oxidative stress

ox-LDL: Oxidized low-density lipoprotein

SOD: Superoxide dismutase

TBARS: Thiobarbituric acid reactive substances

YYIRT-2: Yo-Yo intermittent recovery test level 2

Introduction

Like most team sports, rugby is an intermittent-type activity that involves a high number of repetitive high-intensity efforts interspersed with low-intensity activities or rest ⁽¹⁻³⁾. High-intensity actions are classified as high-speed runs, sprints, acceleration/decelerations, changes of direction, and collision-based activities such as tackling, static holds, scrums, rucks, and mauls ⁽¹⁻⁴⁾.

High-impact collisions received by players, in addition to eccentric damage, elicit extensive tissue trauma ⁽²⁾. The intense physical demands of rugby lead to notable skeletal muscle damage after training and competition ⁽⁵⁾. This skeletal muscle damage is also associated with an inflammatory response characterized by an elevation of proinflammatory cytokines (Interleukin-6, Interleukin-1 β) and other inflammatory blood markers (C-reactive protein (CRP)) in team sports players ^(2, 6-8). This inflammation is

caused not only by muscle mechanical damage itself but also by oxidative stress (OS) induced by exercise ⁽⁹⁾. Indeed, exercise increases free radical production as a consequence of both enhanced oxygen consumption, enzyme activation, and the presence of phagocytic cells activated and recruited to the site of the initial damage ⁽¹⁰⁾. The presence of free radicals may be one of the prime factors in initiating neutrophil infiltration into muscle and the subsequent inflammatory response ⁽¹¹⁾. This increase in free radical production has been also implicated in the oxidative damage to macromolecules ⁽¹²⁾, muscle damage ⁽¹³⁾, and the apparition of overtraining in highly-trained athletes ^(9, 14). Furthermore, exercise-induced OS, muscle damage, and inflammation have been shown to affect neuromuscular function ⁽¹⁵⁾, promote muscle fatigue ^(16, 17), and thus impair exercise performance ⁽¹⁸⁻²⁰⁾. This can lead to an excessive level of accumulated fatigue over the week cycle that may lead to under-performance on match day ⁽²¹⁾.

To prevent and reduce these exercise-related alterations, dietary supplementation may be a promising strategy. Indeed, the consumption of protein, anti-inflammatory, and antioxidant supplements in athletes has increased enormously ^(22, 23). Protein supplements are used by athletes to promote skeletal muscle anabolism and enhance recovery of muscle function and regeneration ^(24, 25). Anti-inflammatory supplements are used to reduce inflammation related to skeletal muscle damage ⁽²⁶⁾ while antioxidants have received interest as a useful tool in preventing or reducing OS and decreasing muscle damage ⁽²⁷⁻²⁹⁾.

Amongst dietary supplements, spirulina (*Arthrospira*) is widely used by athletes. It is a blue-green microalga known for its complex chemical makeup including high protein content (50 to 70% of its dry weight), all the essential amino and fatty- acids, and most of the vitamins and minerals ⁽³⁰⁻³²⁾, which confers to spirulina numerous interesting properties and health benefits such as antioxidant, immunomodulatory, anti-inflammatory, insulin-sensitizing, anti-cancer and anti-viral activities ⁽³²⁻³⁵⁾. The high level of phycocyanin and β -carotene confers to spirulina, antioxidant and anti-inflammatory properties which could be interesting since athletes do not typically provide enough antioxidant vitamins through their diet ⁽³⁶⁻³⁸⁾. Considering intense periods of training and competition, and the deficient nutritional status of elite rugby players, their performance could be impaired and antioxidant system efficiency could be decreased ⁽³⁹⁾.

Literature data is still rare and controversial about spirulina effects on exercise-induced OS and skeletal muscle damage. Indeed, Lu et al. ⁽⁴⁰⁾ showed positive effects of spirulina supplementation in untrained college students, since plasma concentrations of malondialdehyde (MDA) and lactate dehydrogenase (LDH) were significantly lower while the activity of blood superoxide dismutase (SOD) and glutathione peroxidase (GPx) were higher. Antioxidant effects of spirulina were also confirmed in moderately trained individuals by Kalafati et al. ⁽⁴¹⁾ who demonstrated that spirulina supplementation can induce a significant increase in reduced glutathione (GSH) concentration at rest and after exercise, as well as attenuate the exercise-induced increase in lipid peroxidation (TBARS). Contrary to these conclusions, Franca et al. ⁽⁴²⁾ showed that spirulina supplementation was not able to alter the magnitude of skeletal muscle damage or able to reduce OS induced by high volume and intensity of training in cycling athletes with adequate nutritional status. The literature data concerning spirulina supplementation in relation with exercise and/or training in high-level athletes are very rare. Among the three studies exposed above, only one examined elite athletes ⁽⁴²⁾ by measuring changes in parameters only at rest. To our knowledge, no studies have examined the effects of spirulina supplementation in elite team-sport athletes and in response to exercise although they are known for their widespread use of ergogenic aids ⁽⁴³⁾.

Thus, the present study aimed to examine the effects of spirulina supplementation on pro/antioxidant status, inflammation, and muscle damage markers immediately and 24 hours after exhaustive exercise in elite rugby players. We hypothesized that due to its high concentration of proteins and antioxidants, spirulina could reduce exercise-induced OS, inflammation, and skeletal muscle damage in elite rugby players and improve recovery.

Methods

The experimental protocol was approved by a local ethics committee (NOD0172, Tours, France - protocol number 2016-A00233-48). The protocol was conducted in compliance with both Good Clinical Practices and the Declaration of Helsinki. Tests were realized at M2S lab (FINES of 31/05/17; EJ: 350051454; ET: 350051462). After being informed of the objectives, the possible risks, discomforts, and benefits, written and informed consent was obtained from all participants before inclusion.

Participants: Thirty elite male rugby union players belonging to the same French first federal division rugby club (Rennes Étudiants Club « REC ») volunteered to participate in this study. All players completed a clinical examination and medical interview to

determine eligibility. Twenty-two participants (aged 26.0 ± 3.8 years; body weight 99.6 ± 16.6 kg; height 183.1 ± 7.3 cm; body fat 19.5 ± 4.6 %) satisfied the inclusion criteria of being considered healthy in clinical examination, not having any history of surgery, and not taking any kind of dietary supplement for the past 3 months. Exclusion criteria included tobacco use, current intake of spirulina, supplements or anti-inflammatory drugs, known chronic diseases, and allergy to spirulina or any of its components. Only 17 participants finished the study protocol, five players were injured and did not complete all steps of the protocol.

Study design: A randomized, double-blind, placebo-controlled design was used. Participants were assigned to a spirulina group (SPI: $n = 9$), or a placebo group (PLA: $n = 8$). The study consisted of a daily supplementation period during 7 weeks in which the SPI group received capsules with *Arthrospira platensis* certified as safe and healthy, manufactured by Algae Green Value (AGV, Vannes, France). Its composition was described in Table 1. The daily dosage was 5.7g (three capsules before the three daily meals), below the 10g safety dosage recommended by Fox et al. ⁽⁴⁴⁾. The placebo group was given visually identical capsules with isocaloric and isoproteic content composed of 70.3 % egg proteins and 29.7 % carbohydrates.

<< Please insert Table 1 here >>

Rugby players visited the laboratory five times during the study. Anthropometric measurements were taken during the introductory laboratory visit, including height, body mass (BM), and fat mass (FM). Height and BM were collected using a wall-mounted stadiometer (Seca 206, Hamburg, Germany) and electronic scale (Terraillon, Paris, France), respectively. FM percentage was estimated from the sum of skinfolds (biceps brachii, triceps brachii, subscapular, and supra-iliac) monitored with Harpenden skinfold calipers (Baty International, West Sussex, England) using the Siri's equation ⁽⁴⁵⁾.

Blood samples were obtained before (W0) and after seven weeks of supplementation (W7) both at rest (T0) and in response to exhaustive exercise (immediately after (T1), and 24 hours after (T2)). The Yoyo Intermittent Recovery Test Level 2 (YYIRT-2) was used as an exhaustive exercise to induce OS, inflammation, and muscle damage.

Supplementation compliance was assessed by counting the remaining capsules in the provided bottles at the end of the study. Gastrointestinal symptoms were monitored using a questionnaire as described by Bovenschen et al. ⁽⁴⁶⁾. The study design is illustrated in Figure 1.

<< Please insert Figure 1 here

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Experimental procedures: All procedures were conducted during the competitive phase of the season (October-December 2018). All participants actively participated in the rugby-specific training sessions (muscle strength, technical and tactical training) and competitions. They were fully accustomed to the testing procedures used in this research as they routinely performed this test in the club as part of their medical/training follow-up.

Dietary intake: Players had to record their diet for three days including one weekend day at W0 and W7 to establish that they had similar levels of macronutrient and antioxidant intake during the period of data collection. They were asked to maintain their usual dietary habits during the recording period and to be as accurate as possible in recording the amount and type of food and fluid consumed. A color photo exhibition ⁽⁴⁷⁾ of commonly consumed foods and their portion sizes was used to assist in estimating amounts consumed. Diet records were analyzed using the Nutrilog software (Nutrilog® SAS, France).

Training load: Throughout the experiment, the internal training load of all participants was monitored using the session Rate of Perceived Exertion (Session-RPE) method as described by Comyns and Flanagan ⁽⁴⁸⁾ expressed in arbitrary units (AU). 30-minutes (min) after training sessions, players were asked to rate the global intensity of the workout session using the French version CR-10 RPE scale ⁽⁴⁹⁾. The daily training load was evaluated by multiplying the session RPE score by the training duration (min) and the weekly training load was determined by summing the daily training loads each week for each rugby player.

Exhaustive exercise protocol YYIRT-2: Players performed the YYIRT-2 as described by Krstrup et al. ⁽⁵⁰⁾. The YYIRT-2 examines the ability to perform repeated high-intensity exercise bout with an almost maximum aerobic energy production and a high anaerobic energy contribution ^(50, 51). It consists of repeated 40 m (2 x 20 m) runs between markers set 20 m apart, at a progressively increased speed starting from 13 km/h and controlled by audio bleeps from a tape recorder. Between each running bout, the participants had a 10-s of active recovery, consisting of a 10 m (2 x 5 m) walk. When the participant failed to reach the finishing line in time twice or felt unable to continue, the total distance covered (m) was recorded and represented the test result.

Blood sampling: All blood samples were collected from an antecubital vein in two different Vacutainer tubes (EDTA and dry). Players were instructed to fast for 12 h

(overnight) before T0 sampling. After blood sampling, all players were served a standardised breakfast before proceeding to the YYIRT-2 test (1 hour after breakfast). The energy supply corresponded to 18 % of the total caloric intake. The macronutrient repartition was respectively 55%, 15%, 30% for carbohydrates, proteins, and lipids. For a player of 70-90 kg, the standardised breakfast contained: 0.125 L of orange juice, a piece of sugar, 86 g of wholemeal bread, 10 g of butter, 45 g of jam, 200 g of cottage cheese, and one kiwi. At T1, blood was collected 10 min after the end of the exhaustive exercise. At T0, T1, and T2, the EDTA vacutainer was used to evaluate F2 α -isoprostanes (F2-Isop), oxidized low-density lipoprotein (ox-LDL), myeloperoxidase (MPO) in plasma; SOD, GPX, reduced and oxidized glutathione (GSH and GSSG) in whole blood; whereas the dry Vacutainer was used to evaluate CRP, Creatine kinase (CK) and LDH in plasma.

Samples preparation: For GSSG measurement, immediately after blood collection (< 10 s), 100 μ L of whole blood was added to 10 μ L of a scavenger, provided in the GSSG's kit, and were immediately frozen in liquid nitrogen. The other whole blood samples were placed in separated cryotubes (50 μ L for GSH measurement, and 1 mL for each SOD and GPX measurement) and were immediately frozen (< 45 s) in liquid nitrogen. For the rest of the assays, blood was immediately centrifuged at 1500g for 10 min (4° C) to separate plasma (Sigma 2-16K, Osterode, Germany). For F2-Isop, 5 μ L of an ethanolic solution of butylated hydroxytoluene (0.2%) was added to 1 mL of plasma to avoid oxidation. Aliquots were immediately frozen and stored at 80° C until analysis.

Biochemical analysis: A complete blood count was performed on the UniCel[®] DxH800 automated haematology analyser (Beckman Coulter, Miami, FL, USA). GSH/GSSG ratio was determined by the GSH/GSSG-412 kit (Bioxytech, Oxis International Inc., Portland, WA, USA). Enzymatic antioxidant activities of SOD and GPX were determined by the Ransod and Ransel kits (Randox, England), respectively. The ox-LDL levels were determined spectrophotometrically with a competitive enzyme-linked immunosorbent assay (ELISA kit, Immundiagnostik, Germany). Total F2-IsoP was measured by liquid chromatography-mass spectrometry (LC/MS), as described previously ⁽⁵²⁾. The inflammation marker MPO was measured using a colorimetric kit with an enzyme-linked immunosorbent assay (Elisa K 6631, Immundiagnostik, Frankfurt am Main, Germany). CK and LDH were measured with the Cobas[®] 8000 Roche analyser (Roche Diagnostics, Meylan, France) according to the manufacturer's instructions. CRP was measured with latex particle-enhanced immunoturbidimetric assay using the same modular analyzer (Cobas[®] 8000 C701).

Statistical analysis: Data are presented as group mean values and standard deviations (mean \pm SD). After normal distribution was examined using the Shapiro-Wilk-Test, an independent samples t-test was calculated to compare descriptive variables (at W0), internal training load (each week), and dietary intake (at W0 and W7) between-groups. Repeated measures were compared between the two groups using 2 (Group: SPI and PLA) \times 3 (Time: T0, T1, and T2) factorial (two-way) analysis of variance at W0, and W7. If a significant Group \times Time interaction was detected, pairwise comparisons were performed through simple main effect Bonferroni-corrected analysis. The significance level was set at $p < 0.05$. All analyses were performed using SPSS for Windows, Version 20 (IBM Corp: Armonk, NY, USA).

Results

Participants' characteristics and compliance: Participants' baseline characteristics are reported in Table 2. No significant differences were found between SPI and PLA treatments for any of the descriptive parameters. SPI and PLA supplementation compliance was 98.3% and 92.3%, respectively. No adverse effects were reported during or after spirulina supplementation.

<< Please insert Table 2 here >>

Internal training load, and dietary intake: Neither internal training load nor dietary intake showed significant differences between SPI and PLA treatments. Data are shown in Table 3.

<< Please insert Table 3 here >>

The effects of spirulina supplementation: Data collected at W0 for pro/antioxidant status, inflammation and muscle damage markers are not presented in this manuscript since no significant differences were found between groups. To assess spirulina's supplementation ability to prevent the increase of OS, inflammation, and skeletal muscle damage markers after exercise, we compared T0 vs T1 while the ability to accelerate return to baseline values was examined by comparing T1 vs T2 and T2 vs T0. Values at T0 did not differ between the groups for any of the studied parameters.

Pro/antioxidant status before, and after exhaustive exercise: For Pro/antioxidant status markers (SOD, GPX, GSH/GSSG ratio, ox-LDL, and F2-Isop), no significant main effect of "Time" or "Group" and no significant "Group \times Time" interaction was detected for SOD activity and ox-LDL level after spirulina supplementation. A significant main effect of "Time" was observed for only GPX activity and GSH/GSSG ratio. Indeed, GPX

activity significantly decreased ($p<0.05$) in both groups at T2 while GSH/GSSG ratio significantly increased ($p<0.01$) at T1 and significantly decreased ($p<0.01$) at T2 in both groups. However, no significant main effect of “Group” or “Group \times Time” interaction was detected for these parameters. Concerning F2-Isop levels, there was a significant main effect of “Time” and a trend towards significance ($p=0.077$) of “Group \times Time” interaction. Pairwise comparisons showed a significant F2-Isop increase (17.8%) immediately after exercise only in PLA group. No significant change was observed at T2 except a decrease with a trend towards significance ($p=0.074$) in PLA. Moreover, the F2-Isop level at T2 was significantly lower than at T0 in SPI group ($p<0.05$). The evolution of F2-Isop level at T0, T1, and T2 are illustrated in Figure 2A.

Inflammation markers before, and after exhaustive exercise: For inflammation markers (MPO and CRP), no significant main effect of “Time” or “Group” and no significant “Group \times Time” interaction were detected for MPO after spirulina supplementation. However, there was a significant main effect of “Time” and “Group \times Time” interaction for CRP. As shown in Figure 2B, plasma CRP increased significantly at T1 in PLA (10.4%) with no change in SPI group. Moreover, spirulina supplementation accelerated the return to baseline values given that plasma CRP decreased significantly at T2 in SPI group (-27.5%) with a lower level compared to T0 ($p<0.01$) whereas, no significant change was observed in PLA compared with T1.

<< Please insert Figure 2 here >>

Skeletal muscle damage markers before, and after exhaustive exercise: For muscle damage markers (LDH and CK), no significant main effect of “Group” and no significant “Group \times Time” interaction was detected for LDH after spirulina supplementation. However, there was a significant main effect of “Time” with plasma LDH increasing significantly at T1 ($p<0.01$) and then significantly decreasing at T2 ($p<0.01$) in both groups without significant difference between groups. For CK, a significant main effect of “Time” and “Group \times Time” interaction was found. As shown in Figure 2C, plasma CK increased significantly at T1 in PLA (15.5%) with no change in SPI group. Moreover, spirulina supplementation accelerates the return to baseline values given that plasma CK decreased significantly at T2 in SPI (-34.2%) with a lower level compared to T0 ($p<0.001$) whereas, no significant change was observed in PLA compared with T1. Table 4 shows the values for parameters that did not show any significant “Group \times Time” interaction at T0, T1, and T2.

<< Please insert Table 4 here >>

Discussion

This study is the first to examine the effects of spirulina supplementation on exercise-induced OS, inflammation, and skeletal muscle damage markers in elite athletes. Based on the markers used in this study, our results report that spirulina supplementation potentially prevents exercise-induced lipid peroxidation, inflammation, skeletal muscle damage and may accelerate the recovery of some of these markers (F2-Isop, CRP, and CK).

Whereas very few studies evaluated spirulina supplementation in athletes, numerous publications examined the properties of this microalga in the prevention and treatment of various diseases. These studies have shown that its unusually high nutritional values confer to spirulina diverse beneficial health effects such as antioxidant, anti-inflammatory, insulin-sensitizing, immunomodulatory, anti-cancer, and anti-viral activities⁽³²⁻³⁵⁾. All these properties shown in patients could be interesting for team sports players considering OS, inflammation, and skeletal muscle damage that may be induced by intense training and competition^(2, 5, 6, 53).

Intense exercise, training and competition induce OS, inflammation, and skeletal muscle damage which could impair exercise performance by affecting neuromuscular function⁽¹⁵⁾, decreasing muscle strength^(54, 55), and promoting muscle fatigue^(16, 17). Since spirulina is known for its high protein content including all the essential amino acids⁽³⁰⁻³²⁾, it could be of great interest for athletes especially at high level. Moreover, intense training has been shown to decrease the availability of essential amino acids, which may slow the rate of tissue repair and growth⁽⁵⁶⁾. Athletes need to ingest enough high-quality protein to maintain essential amino acid availability⁽⁵⁷⁾, to reduce skeletal muscle damage^(58, 59), and to enhance recovery⁽²⁴⁾. Spirulina's composition is interesting for this population because in addition to amino acids it contains many compounds with antioxidant and anti-inflammatory virtues favorable to the process of muscle regeneration and skeletal muscle damage reduction⁽⁶⁰⁾.

To the best of our knowledge, our study is the first that used the YYIRT-2 as an exhaustive exercise to induce OS, inflammation, and skeletal muscle damage. We chose the YYIRT-2 test because it evaluates the abilities to perform intense intermittent exercise (with a large anaerobic component in combination with a significant aerobic contribution) and to recover quickly, which are essential components during the intense periods of Team-sports games⁽⁵¹⁾. The YYIRT-2 is an effective testing tool able to

induce OS, inflammation, and skeletal muscle damage in elite athletes because our results showed an increase in F2-Isop, CRP, and CK levels for all participants immediately after exercise at W0. Indeed, top-level athletes are well accustomed to this type of solicitation during their habitual training and competition.

Concerning the antioxidant effects of spirulina, we are the first to measure its effects on ox-LDL and F2-Isop as markers of exercise-induced lipid peroxidation in humans. F2-Isop is considered as the gold standard biomarker of lipid peroxidation⁽⁶¹⁾ while ox-LDL is believed to be one of the major factors involved in the pathogenesis of atherosclerosis⁽⁶²⁾. Our results did not demonstrate any significant change for ox-LDL (Table 4) but showed a significant main effect of “Time” and a trend towards significance of “Group × Time” interaction with F2-Isop significantly increased ($p < 0.05$) immediately after exercise only in PLA group. Moreover, the F2-Isop level at 24h after exercise was significantly lower ($p < 0.05$) than at rest in SPI group (Figure 2A). These findings are in accordance with other studies^(40, 41) suggesting that spirulina supplementation prevents exercise-induced lipid peroxidation. In fact, Lu et al.⁽⁴⁰⁾ showed that plasma concentrations of MDA were significantly decreased after spirulina supplementation. They reported a less increase in the placebo group without significant difference between groups. The same results were reported in animal studies^(63, 64) showing a less increase in MDA level after spirulina supplementation showing its ability to inhibit lipid peroxidation. In the same context, although there has been some general concern about the validity of the TBARS assay because of its lack of specificity concerning lipid peroxidation⁽⁶⁵⁾, Kalafati et al.⁽⁴¹⁾ detected a significant interaction in moderately trained men, with TBARS levels increasing after exercise in the placebo group but not after spirulina supplementation. In contrast, Franca et al.⁽⁴²⁾ reported no effects of spirulina supplementation on the MDA level in elite cycling athletes subjected to high volume and intensity of training. It should be noted that the measurements in this study⁽⁴²⁾ were carried out at rest and not after exhaustive exercise. For the other markers of pro/antioxidant status (GSH/GSSG, SOD, and GPX), no significant between-group difference was found in our study (Table 4) as already described by Kalafati et al.⁽⁴¹⁾ in moderately trained, and by Franca et al.⁽⁴²⁾ in highly trained participants but only at rest. Contrary results were found by Lu et al.⁽⁴⁰⁾ showing a significantly higher post-exercise GPX level after spirulina supplementation in untrained students. Similarly to Lu et al.⁽⁴⁰⁾, animal studies showed a significant increase in post-exercise GPX⁽⁶³⁾, and SOD^(63, 64) activity after spirulina supplementation compared to placebo recommending this microalga to enhance the antioxidant status and to inhibit OS induced by acute

exhaustive exercise. These contradictory results could be attributed to the different participants' training levels since it is well known that antioxidant enzymes are upregulated by training^(66, 67) thus making the antioxidant effect of spirulina more difficult to detect in this population.

For inflammation markers, our results showed no significant change for MPO after spirulina supplementation (Table 4). In contrast, plasma CRP increased significantly ($p < 0.05$) in PLA immediately after exercise without any change in SPI group. Moreover, no significant change was observed in PLA 24h after exercise whereas plasma CRP decreased significantly ($p < 0.01$) in SPI with a lower level compared to rest value (Figure 2B). These results reflect the effect of spirulina supplementation to prevent inflammation induced by exhaustive exercise and to accelerate the return to baseline values. To our knowledge, there are no studies that examined the effects of spirulina supplementation on exercise-induced inflammation markers in humans. Anyway, our findings are consistent with the results of a recent animal study investigating spirulina supplementation on inflammation promoted by strength training⁽⁶⁸⁾, in which the concentration of CRP decreased significantly after eight weeks of supplementation.

Concerning muscle damage markers, our findings did not show any significant difference between groups in LDH level (Table 4). However, plasma CK increased significantly ($p < 0.001$) only in PLA immediately after exercise without any change in SPI group. Moreover, no significant change was observed in PLA 24h after exercise whereas plasma CK decreased significantly ($p < 0.001$) in SPI with a lower level compared to rest value (Figure 2C). These results reflect the effect of spirulina supplementation preventing exercise-induced muscle damage and accelerating damage recovery. The effects of spirulina supplementation on exercise-induced muscle damage in humans are not well documented in the scientific literature. Lu et al.⁽⁴⁰⁾ reported a decrease in CK level in the SPI group while it increased in the placebo group but without significant difference between groups. Kalafati et al.⁽⁴¹⁾ reported an increase of CK activity 24 and 48 h after exercise in both groups without significant difference while Franca et al.⁽⁴²⁾ did not show any significant change at rest for LDH or CK levels in high-level cycling athletes. Franca et al.⁽⁴²⁾ concluded that the administration of Spirulina does not interfere in the magnitude of skeletal muscle damage in cycling athletes that practice intense training, and explained their results by the fact that athletes had an adequate nutritional status regarding macronutrients.

Spirulina contains several active ingredients, notably phycocyanin and β -carotene that have potent antioxidant and anti-inflammatory activities. As antioxidant mechanisms of action described in the literature ^(34, 35), phycocyanin has the ability to scavenge free radicals, decrease nitrite production, suppress inducible nitric oxide synthase (iNOS) expression, and inhibit lipid peroxidation. In the same way, it was found that β -carotene protected against singlet oxygen-mediated lipid peroxidation, inhibited the production of nitric oxide and prostaglandin E(2), and suppressed the expression of iNOS and cyclooxygenase-2 (COX-2). Besides, phycocyanin and β -carotene inhibit proinflammatory cytokine formation, such as tumor necrosis factor α (TNF α), suppresses COX-2 expression, and decreases prostaglandin E(2) production as anti-inflammatory activities. It has been also reported to suppress the activation of nuclear factor- κ B (NF- κ B) through preventing degradation of cytosolic inhibitor of nuclear factor κ B- α (I κ B- α) and modulate the mitogen-activated protein kinase (MAPK) activation pathways.

Conclusion

Based on the markers used in this study, our results report that spirulina supplementation potentially prevents exercise-induced lipid peroxidation, inflammation, skeletal muscle damage and may accelerate the recovery of some of these markers. Although the favourable results of our study, we recognise that there are limitations. The discrepancy in effect on markers and the small sample size would limit the validity and generalizability of our findings. Furthermore, we only explored a small number of biomarkers and therefore cannot know whether other relevant markers would have been affected by spirulina in a consistent way. The high density of the weekly rugby schedule results in a limited time for recovery between training sessions, which is associated with a decrease in performance. OS, muscle damage, and the consequent inflammation from rugby play a primary role in the decreased capability to perform. Based on our findings, we recommend spirulina supplementation to athletes who perform a high training load in order to reduce the magnitude of OS, inflammation, and muscle damage which could help reduce performance losses and accelerate recovery after training/competitions throughout the season. The mechanisms of action behind the above-mentioned effects of spirulina related to exercise are poorly understood. Besides, the differences in the study designs and the lack of research in the literature regarding the effects of spirulina supplementation in athletes make it difficult to make practical recommendations. More investigations are needed to elucidate this.

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Competing interests

The authors declare that they have no competing interests.

AUTHORS’ CONTRIBUTION

Chaouachi M: Data curation, Formal analysis, Investigation, Methodology, Statistics, Validation, Writing - original draft - review & editing, Validation;

Gautier S, Carnot Y, Guillemot P, Pincemail J, Moison Y, and Collin T: Data curation, Investigation;

Groussard C, and Vincent S: Conceptualization; Formal analysis, Funding acquisition, Investigation, Methodology, Project administration, Supervision, Writing – review & editing, Validation;

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Table 1. Composition of *Arthrospira platensis* produced by AGV (100 g).

	Unity	Amount		Unity	Amount
Energy			Vitamins		
	kJ	1530	Vitamin A	µg	<20
	kcal	363	β-carotene	mg	24,902
Fat			Vitamin C	mg	<2,5
Total fat	g	6,3	Vitamin B3	mg	8,5
Saturated fat	g	3,8	Vitamin B5	mg	0,05
Unsaturated fat	g	2,5	Vitamin B6	mg	0,09
Monounsaturated fat	g	0,4	Vitamin B9	µg	156
Polyunsaturated fat	g	2	Vitamin B12	µg	61,3
Omega 3	g	0,008	Vitamin E	mg	6,84
Omega 6	g	0,9	Minerals		
Carbohydrates			Sodium	mg	1338,2
Total Carbohydrates	g	16,5	Phosphorus	mg	1353,8
Dietary Fiber	g	7,4	Calcium	mg	310,4
Proteins and amino acids			Copper	mg	1,83
Proteins	g	63,7	Iron	mg	76,43
Leucine	g	5,56	Magnesium	mg	434,2
Isoleucine	g	3,47	Manganese	mg	7,06
Valine	g	4,02	Potassium	mg	1932
			Zinc	mg	7,43

Table 2. Participant characteristics.

	SPI (n=9)	PLA (n=8)	<i>p</i> -value
Age (year)	25.2 ± 2.7	25.9 ± 3.3	0.660
BM (kg)	97.3 ± 13.3	101.1 ± 19.0	0.635
Height (cm)	182.3 ± 4.7	182.7 ± 6.1	0.894
FM (%)	19.2 ± 4.9	19.9 ± 4.4	0.728
FM (kg)	19.2 ± 6.8	20.7 ± 7.5	0.661
LBM (kg)	78.2 ± 7.3	80.4 ± 12.4	0.649
Distance YYIRT-2 (m)	426.7 ± 385.0	385.0 ± 95.5	0.478

Values are presented as mean ± standard deviation. PLA, placebo; SPI, Spirulina; BM, body mass; FM, Fat Mass; LBM, lean body mass; YYIRT-2, Yoyo intermittent recovery test-level 2.

Table 3. Participants' daily dietary intake and internal training load.

		SPI (n=9)	PLA (n=8)	<i>p</i> -value (T-test)	<i>“Group × Week” interaction</i>
Caloric intake (kcal/d)	W0	2662.9 ± 522.1	2901.7 ± 651.4	0.415	0.139
	W7	2817.5 ± 559.5	2705.5 ± 544.2	0.682	
Carbohydrates (g/kg/d)	W0	2.81 ± 0.97	2.88 ± 1.09	0.891	0.084
	W7	3.13 ± 1.01	2.67 ± 0.81	0.316	
Proteins (g/kg/d)	W0	1.40 ± 0.40	1.69 ± 0.72	0.306	0.167
	W7	1.45 ± 0.37	1.63 ± 0.72	0.541	
Fat (g/kg/d)	W0	1.18 ± 0.31	1.13 ± 0.34	0.751	0.893
	W7	1.20 ± 0.31	1.16 ± 0.44	0.851	
Internal training load (AU)	W1	1497.8 ± 348.5	1210.7 ± 734.0	0.310	0.411
	W2	1363.7 ± 347.5	1053.6 ± 840.0	0.325	
	W3	1224.4 ± 235.8	1339.4 ± 362.0	0.445	
	W4	690.0 ± 206.2	768.8 ± 120.0	0.359	
	W5	1360.4 ± 364.8	1392.1 ± 542.6	0.888	
	W6	1639.6 ± 295.8	1630.5 ± 431.1	0.960	

Values are presented as mean ± standard deviation.

Table 4. Effects of supplementation on pro/antioxidant status, inflammation and skeletal muscle damage.

		T0	T1	T2	<i>“Group × Time” interaction</i>
<i>Pro/antioxidant status</i>					
SOD (UI/g Hb)	PLA	31.65 ± 2.65	31.64 ± 3.13	31.85 ± 3.70	0.787
	SPI	34.06 ± 3.07	34.44 ± 2.68	34.11 ± 2.56	
GPX (UI/g Hb)	PLA	509.93 ± 57.55	504.06 ± 56.71	483.00 ± 58.36	0.347
	SPI	553.98 ± 90.62	562.41 ± 106.36	549.25 ± 106.85	
GSH/GSSG	PLA	197.25 ± 190.16	531.29 ± 210.85	207.86 ± 89.60	0.836
	SPI	205.25 ± 127.57	467.38 ± 280.83	174.14 ± 103.59	
Ox-LDL (U/L)	PLA	68.75 ± 29.97	64.88 ± 24.39	66.25 ± 24.15	0.803
	SPI	57.44 ± 13.12	58.11 ± 22.73	53.33 ± 17.59	
<i>Inflammation</i>					
MPO (ng/mL)	PLA	53.13±11.68	54.13±12.77	53.00±11.10	0.759
	SPI	51.63±13.81	51.75±11.47	49.00±10.43	
<i>Skeletal muscle damage</i>					
LDH (U/L)	PLA	168.57 ± 41.64	197.29 ± 58.84	163.86 ± 41.77	0.871
	SPI	186.29 ± 24.00	209.86 ± 17.04	175.00 ± 27.30	

Values are presented as mean ± standard deviation. T0, before exercise; T1, immediately after; T2, 24h after.

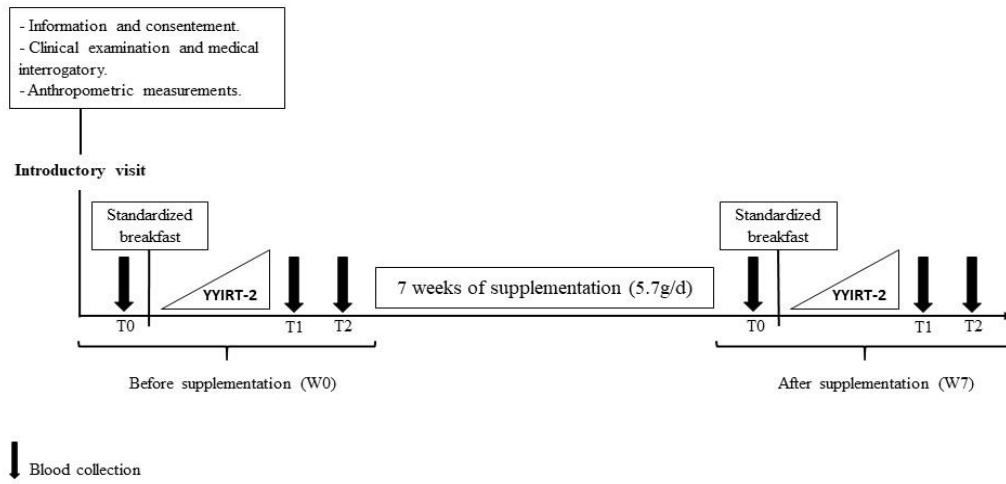


Figure 1: Study design.

W0, before supplementation; W7, after supplementation; T0, before exercise; T1, immediately after;

T2, 24h after; YYIRT-2, Yoyo intermittent recovery test-level 2.

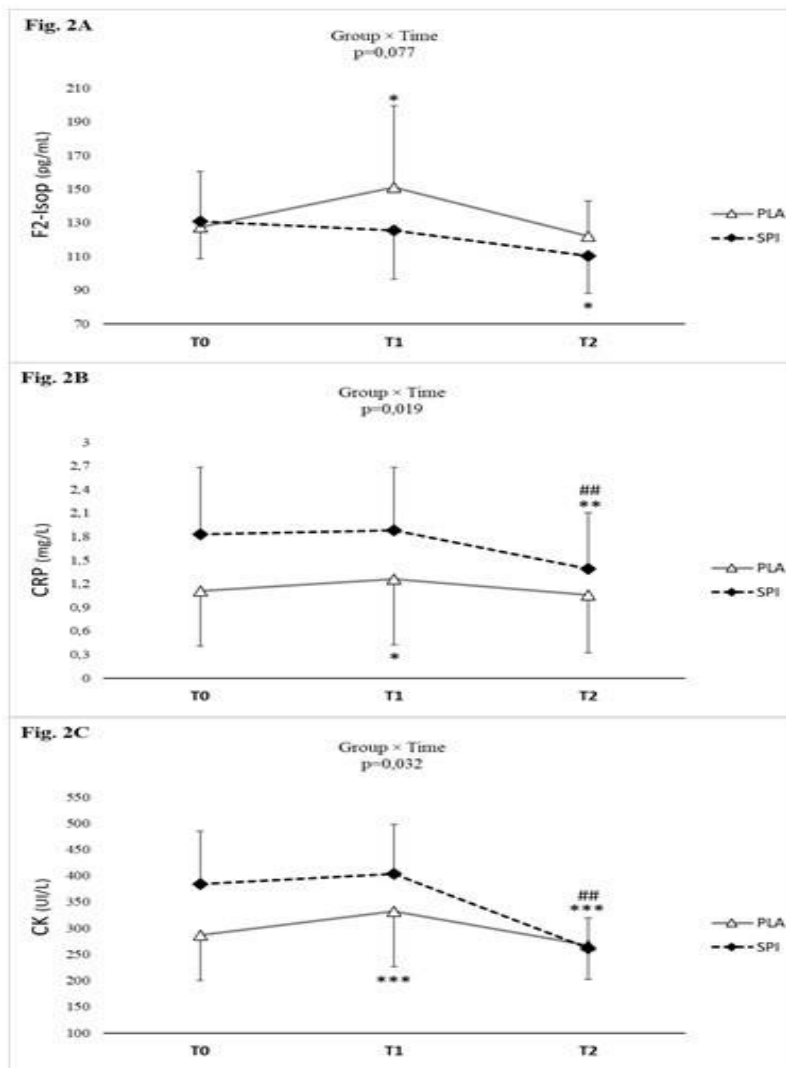


Figure 2: Changes in plasma F2-Isop (Fig. 2A), CRP (Fig. 2B) and CK (Fig. 2C) levels after seven weeks of supplementation determined before (T0), immediately (T1) and 24h (T2) after exercise.

Values are reported as mean \pm SD. * Significant difference with T0 at $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. ## Significant difference with T1 at $p < 0.01$.