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# Antioxidant capacity and athletic condition of endurance horses undergoing nutraceutical supplementation

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ABSTRACT

Endurance is an equestrian discipline that primarily relies on aerobic metabolism. Intense aerobic exercise produces reactive oxygen species due to an imbalance between oxidant and antioxidant substances, known as oxidative stress, which may reduce athletic performance. This study evaluated the effects of a feed supplement containing natural antioxidants and omega-3 fatty acids on the blood antioxidant activity and the athletic condition of endurance horses undergoing an exercise test. Twelve Arabian endurance horses were randomly assigned to treatment or control groups. At T0, blood lactate, whole blood and red blood cells (RBC) antioxidant capacity were assessed. The horses performed an exercise test with heart rate monitoring. After 30 min, blood lactate, antioxidant capacity and serum creatine kinase (CK) were measured. The treatment group received the dietary supplement for 21 days, while controls maintained their diet. Then, the protocol was repeated (T1). Variables were compared within and between groups through two-way ANOVA and post-hoc tests. Significant time*group effects were observed for serum CK ($p = 0.026$), RBC antioxidant capacity at rest ($p = 0.034$) and post-exercise ($p = 0.019$). At T1, in treatment group, CK was lower than controls ($p = 0.006$), while RBC antioxidant capacity increased at rest ($p = 0.037$) and after exercise ($p = 0.006$) compared to T0. The dietary supplement showed efficacy in enhancing RBC antioxidant capacity, and it could be beneficial for horses engaged in intense aerobic exercise.

## 1. Introduction

Endurance riding is a popular equestrian discipline, demanding a significant physical exertion that can be highly challenging for the cardiovascular and the musculoskeletal systems of horses [1,2]. Endurance exercise tests resistance and speed, primarily relying on aerobic metabolism [3] but may also involve phases of anaerobic exercise [4]. The intense aerobic activity induces an increased reactive oxygen species (ROS) production, that may result in in an imbalance between oxidants and antioxidants [5–7]. This condition was observed both in human [8–12] and horses [5,7,13–18]. While mild exercise with moderate ROS production has beneficial effects, such as an enhanced adaptive response to training and improved antioxidant capacity, high levels of ROS production may lead to muscular dysfunction, increased fatigue, prolonged recovery, and reduced athletic performance [10,11,12]. In endurance horses, intense exercise has been associated with systemic inflammation and increased ROS production, especially from mitochondria, associated with skeletal muscle damage [6,19–24]. Indeed, increased muscle membrane leakage has been correlated with higher concentrations of oxidant substances in intensely trained horses [5,15,17,25]. Chronic inflammation and repetitive skeletal muscle tissue trauma may contribute to poor health and sports performance impairment [23,26–28]. The attenuation of oxidative stress may help in protecting horses from ROS-associated damage and, consequently, for promoting optimal athletic condition [7]. Endogenous antioxidant systems include both enzymatic (i.e., superoxide dismutase, catalase, glutathione peroxidase, etc.) and non-enzymatic (i.e., vitamin E, vitamin C, vitamin A, micronutrients, etc.) defenses that protects tissues from oxidative stress, by neutralizing ROS [13,14,20,29]. However, when endogenous defenses may be overwhelmed by oxidative stressors, exogenous dietary antioxidants may act synergistically to protect tissues from ROS-mediated injury [16,28]. On this basis, the use of nutraceutical supplements containing antioxidant substances has attracted considerable attention due to their potential to minimize physical stress, reduce muscular damage, and improve sports performance in both human [9,10,12,13] and equine athletes [19,30–35]. Dietary

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supplementation with a combination of antioxidant molecules has been shown to have synergistic effects and is recommended rather than supplementation with a single antioxidant [5,8,25,36]. Moreover, the dietary supplementation with long chain omega-3 polyunsaturated fatty acids, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acid, have extensively demonstrated to have anti-inflammatory properties [30,32,34,37–40], and several studies reported their effects on performance enhancement and muscle protection [30,31,34,36,40,41]. Supplementation with natural antioxidants and long-chain omega-3 fatty acids could therefore be of great interest to endurance horses. For these reasons, a nutraceutical feed supplement specifically designed to protect the muscles and promote athletic recovery in sport horses has been tested. The feed supplement contains vitamin C and extracts of Schizochytrium microalgae, rich in DHA [38,40,42], along with Vitis vinifera [9,10,43–45], Spirea ulmaria [46,47] and Filipendula ulmaria [29,46,48–50], providing abundant polyphenols. The hypothesis of the present study was that the nutraceutical ingredients in this supplement would exhibit antioxidant and anti-inflammatory effects in endurance horses undergoing intense aerobic exercise. Therefore, we aimed to investigate the effect of a 21-day dietary supplement on the total antioxidant capacity, exercise-induced muscular membrane leakage, and the athletic physiological parameters of endurance horses in training.

## 2. Materials and methods

### 2.1. Ethical approval

Ethical approval for the study was granted by the Animal Welfare Organization of the University of Milan (Protocol Number OPBA_16_2022). Written informed consent for the inclusion of patients in the present study was obtained from horse owners and/or delegated holders

### 2.2. Study population

Twelve endurance horses in full training were included in the present study. All horses came from the same stable, were housed in individual boxes with paddock access, fed daily with hay and commercial pelleted diet and subjected to the same training regimen, which consisted in aerobic exercise (walk, trot and light canter) for two hours three times a week. A complete preliminary physical examination and blood cell count were performed to exclude obvious clinical diseases, and horses were classified as healthy at enrollment.

### 2.3. Study design

On the first day (T0), enrolled horses underwent a comprehensive protocol including evaluations at rest, during exercise, and post-exercise. At rest, a 9 mL blood sample was collected, and blood lactate concentration was immediately assessed on a 3 μl blood drop, using a portable lactate monitor (Lactate Pro 2; Cosmed, Italy). The remaining blood sample was collected in a sterile tube containing Ethylenediaminetetraacetic acid (EDTA) and stored at 4 °C for later antioxidant capacity test. Horses were then harnessed with their standard equipment, wearing a continuous heart rate monitor (Equimetre; Arioneo, France). Once ready, horses were ridden on a 1100-meter-long racing track, undergoing an exercise test including a 15-minutes warm-up at trot, and 60 min gallop at 20 km/h (30 min left-handed and 30 min right-handed). Mean and maximum heart rate (HR) reached during exercise were recorded. After the end of exercise, horses were cooled down and showered. The HRs at 5-, 10-, 15-, and 30-minutes post-exercise were recorded to evaluate the recovery times. At 30 min post-exercise, other blood samples were collected: blood lactate concentration was assessed as described above, a 9 mL sample was collected in a sterile plain tube for later serum creatine kinase (CK) measurement, and a 9 mL sample was stored in a sterile tube containing EDTA for antioxidant capacity test. To evaluate the blood lactate production induced by exercise, Δ lactate was calculated as the difference between post- and pre-exercise blood lactate concentrations. After 21 days (T1), the protocol was repeated. Environmental data of temperature and relative humidity during the tests were obtained from a local meteorological association (Associazione Meteopassione APS, Italy).

### 2.4. Dietary treatment

The horses were randomly assigned to two groups: control group, which had no dietary modifications, and treated group, that received 100 grams/day of the dietary supplement containing omega 3 fatty acids and natural antioxidants mixture (Algaphyt; Equiplanet by TecnoZoo, Italy), administered orally within the concentrate feed, for 21 days. The ingredients, analytical constituents and additives of the dietary supplement, as reported by the producer, are listed in Table 1. Training regimen, housing, and management remained unchanged in both groups.

### 2.5. Antioxidant capacity test

The antioxidant capacity of whole blood and red blood cells (RBC) was assessed by the Kit Radicaux Libres (KRL) test (Laboratoires Spiral, France) [51]. The KRL test is a bio-logical test, recently validated in horses, which evaluates blood antioxidant defenses and resistance to the hemolysis induced by a thermo-controlled free radical attack [52]. Whole blood and RBC samples, diluted in an isotonic saline solution, were exposed to organic free radicals generated at 37° from the thermal decomposition of a solution of 2.2′–azobis (2-amidinopropane) dihydrochloride (AAPH; Kirial International, France). The optical density decay at 450 nm was measured using a 96-well microplate reader (KRL Reader; Kirial International, France) to record hemolysis. For each well, absorbance measurements were undertaken 75 times, once every 150 seconds. The resulting KRL values represent the time needed to reach 50 % of maximal hemolysis: this time, expressed in minutes, is defined as half-hemolysis time, and reflects the whole blood and RBC resistance to free radicals attack [51,52].

### 2.6. Statistical analysis

Statistical analysis was conducted using a commercially available statistical software package (Prism 10; GraphPad Software, USA). Data distribution was assessed by Shapiro-Wilk test and descriptive statistics were performed. Normally distributed data are expressed as mean ±standard deviation, while non-normally distributed data are reported as median and interquartile ranges. Environmental temperature and relative humidity were compared between T0 and T1 by Wilcoxon rank test. Age and sex were compared between groups using, respectively, the unpaired t test and the Fisher's exact test. The effects of the supplement administration on all variables (resting and post-exercise blood lactate, resting and post-exercise whole blood and RBC KRL values, post-exercise serum CK, and mean, maximum and post-exercise HRs) were evaluated by two-way repeated-measures ANOVA, investigating group, time, and

**Table 1**
Ingredients, analytical components and additives of the nutraceutical supplement, as reported by the producer.

| | |
|---|---|
| Ingredients | Alfalfa, extract of algae of the *Schizochytrium* genus (20 %), phenols and concentrated polyphenols obtained from hydrolysed lignocellulose, calcium carbonate, products obtained from the processing of vegetables (*Vitis vinifera, Spiraea ulmaria, Filipendula ulmaria*), wheat feed flour. |
| Analytical components | Raw protein 8.3 %, raw fats 12.3 %, raw fibre 14.1 %, raw ash 24.7 %, methionine 0.09 %, L-lysine 0.25 %, sodium 0.03 %, calcium 7.5 % |
| Additives per kg | Vitamin C 10,000 mg |

group*time interaction effects, besides subject-related variations. When statistical significance was observed, post-hoc Fisher's LSD test was performed. Statistical significance was set at $P < 0.05$.

## 3. Results

### 3.1. Horses

Of the 12 horses initially enrolled, 11 completed the study as one was lost at follow-up due to acute lameness unrelated to the trial. The final study population comprised 6 horses included in the treatment group and 5 in the control group. All were Arabian horses, aged between 7 and 15 years (mean 6.5 ± 3.6 years), including 5 mares (45.5 %) and 6 geldings (55.5 %). No statistical differences were observed for age (treatment: 10.2 ± 2.1 years; control: 11 ± 2.2 years) nor for sex distribution (treatment: 4 mares, 2 geldings; control: 1 mare, 4 geldings) between the two groups. At both T0 and T1, clinical examinations and cell blood count results were within normal limits (Table 2). No adverse events were observed during the 21-day supplementation period and the product was readily consumed by all horses in the treatment group. No significant differences in environmental temperature and relative humidity were detected between T0 (temperature 20.9 °C, 12.4 – 26 °C; humidity 66.3 %, 57 – 72.3 %) and T1 (temperature 24.3 °C, 11.9 – 25.8 °C; humidity 64 %, 61.7 – 76 %): therefore, their effects on the horses-related variables were considered equal at both timepoints.

### 3.2. Physiological variables

The physiological variables measured at baseline, during and/or after exercise (blood lactate, heart rate, CK), in the treatment and control group, at T0 and T1, are displayed in Table 3. No significant group or time effects were observed for any of the blood lactate and heart rate variables. A significant time*group effect was observed for post-exercise serum CK ($P = 0.026$). In the treatment group, CK was significantly lower at T1 compared to T0 ($P = 0.006$). No differences within the control group were observed between T0 and T1, nor between groups at any timepoint. Moreover, a significant subject-related variation was observed for mean HR recorded during the exercise test ($P < 0.001$) and post-exercise serum CK ($P = 0.039$).

**Table 2**
Resting physical examination parameters and hematology results, expressed as mean±standard deviation, obtained at T0 and T1 in the treatment and groups.

| Variable | Treatment Group (n = 6) | | Control Group (n = 5) | |
|---|---|---|---|---|
| | T0 | T1 | T0 | T1 |
| Heart rate (bpm) | 36.7 ± 1.63 | 36.7 ± 3.01 | 36.8 ± 5.22 | 36 ± 4.9 |
| Respiratory rate (bpm) | 17.3 ± 3.27 | 16.7 ± 3.01 | 16 ± 2.83 | 15.2 ± 4.38 |
| Rectal temperature (°C) | 37.6 ± 0.52 | 37.7 ± 0.43 | 37.4 ± 0.31 | 37.6 ± 0.53 |
| Red Blood Cells (x106/μL) | 7.81 ± 0.29 | 7.85 ± 0.31 | 8.35 ± 0.56 | 8.57 ± 0.66 |
| Hemoglobin (gr/dL) | 13 ± 0.5 | 13.3 ± 0.79 | 13.7 ± 1.32 | 13.8 ± 1.18 |
| Hematocrit (%) | 35.6 ± 1.36 | 35.5 ± 1.85 | 37.2 ± 3.16 | 37.6 ± 3 |
| White Blood Cells (x103/μL) | 8.3 ± 1.29 | 9.27 ± 1.64 | 7.43 ± 1.09 | 7.59 ± 1.36 |
| Granulocytes (x103/μL) | 5.31 ± 0.8 | 5.6 ± 1.19 | 3.94 ± 0.69 | 4.54 ± 0.57 |
| Lymphocytes (x103/μL) | 2.5 ± 0.76 | 3 ± 0.54 | 2.97 ± 0.47 | 2.76 ± 0.75 |
| Monocytes (x103/μL) | 1.16 ± 1.61 | 0.63 ± 0.26 | 0.52 ± 0.25 | 0.3 ± 0.17 |
| Platelets (x103/μL) | 124 ± 63.5 | 129 ± 63.1 | 133 ± 38.6 | 159 ± 65.1 |

**Table 3**
Blood lactate concentrations at rest, at 30 min post-exercise, and Δ blood lactate concentrations, heart rate (HR) values recorded during exercise (mean and maximum) and at 5-, 10-, 15-, and 30-minutes post-exercise, and serum creatine kinase (CK) measured at 30 min post-exercise, in the treatment and control groups, at T0 and T1. Data are expressed as mean±standard deviation. Statistical significance is shown as ** ($P < 0.01$).

| Variable | Treatment Group (n=6) | | Control Group (n=5) | |
|---|---|---|---|---|
| | T0 | T1 | T0 | T1 |
| Lactate at rest (mmol/L) | 0.85 ± 0.18 | 1.05 ± 0.37 | 0.76 ± 0.22 | 0.86 ± 0.28 |
| Lactate 30 min (mmol/L) | 1.53 ± 0.44 | 1.38 ± 0.40 | 1.46 ± 0.64 | 1.46 ± 0.74 |
| Δ Lactate (mmol/L) | 0.68 ± 0.38 | 0.33 ± 0.36 | 0.70 ± 0.47 | 0.46 ± 0.53 |
| Mean HR (bpm) | 128.0 ± 8.85 | 127.0 ± 7.87 | 128.6 ± 13.4 | 127.4 ± 8.59 |
| Maximum HR (bpm) | 144.2 ± 7.41 | 145.8 ± 8.13 | 146.4 ± 15.5 | 151.2 ± 6.54 |
| HR 5 min (bpm) | 58.8 ± 6.97 | 59.7 ± 10.9 | 59.6 ± 5.03 | 52.0 ± 2.35 |
| HR 10 min (bpm) | 47.0 ± 6.51 | 52.0 ± 6.29 | 46.2 ± 4.38 | 50.4 ± 6.27 |
| HR 15 min (bpm) | 42.2 ± 5.35 | 46.7 ± 5.65 | 44.6 ± 3.98 | 47.0 ± 5.15 |
| HR 30 min (bpm) | 40.0 ± 6.78 | 41.0 ± 7.38 | 41.0 ± 1.58 | 42.8 ± 6.6 |
| CK 30 min (IU/L) | 330.8 ± 121.8** | 254.8 ± 63.5** | 258.4 ± 27.2 | 271.2 ± 47.8 |

### 3.3. Antioxidant capacity

The baseline (at rest) and 30-minutes post-exercise KRL values of whole-blood and RBC, in the treatment and control groups, at T0 and T1, are shown in Fig. 1. For baseline whole-blood KRL values, a significant group effect was observed ($P = 0.029$), with horses in the treatment group showing lower values compared to the control group at T0 ($P = 0.011$). However, no significant time nor time*group effects were observed. Concerning post-exercise whole-blood KRL values, no significant time nor group effects were observed. Conversely, significant group ($P = 0.038$) and time*group effects ($P = 0.034$) were observed for baseline RBC KRL values. Horses in the treatment group showed a lower baseline RBC KRL value at T0, compared to horses in the control group ($P = 0.005$). Moreover, in the treatment group, baseline RBC KRL values significantly increased at T1 compared to T0 ($p = 0.037$). Similarly, a significant time*group effect was observed for post-exercise RBC KRL values ($P = 0.019$). Specifically, post-hoc analysis showed a significant increase at T1 in the treatment group compared to T0 ($P = 0.006$). Finally, significant subject-related variations were observed for post-exercise whole-blood KRL values ($P = 0.016$), and baseline ($P = 0.006$) and post-exercise ($P = 0.005$) RBC KRL values.

## 4. Discussion

After 21 days of supplementation, the horses fed the nutraceutical supplement showed an enhanced antioxidant capacity of RBC, both at rest and after exercise, along with a reduced post-exercise muscular membrane leakage, suggesting the beneficial effects of the investigated feed supplement for conditioned endurance horses.

The dietary supplement contained the microalgae extract of the genus *Schizochytrium*, as an anti-inflammatory substance. These microalgae accumulate a substantial amount of DHA (over 35 % of the total fatty acid content), with variable concentrations of EPA depending on the species [38,40,42]. These long-chain omega-3 polyunsaturated fatty acids are integral components of the cellular phospholipid membranes [32], with proven health benefits, mainly in reducing inflammatory responses [30,38–40]. While the exact mechanisms underlying this effect are not completely clarified [42], it has been hypothesized that

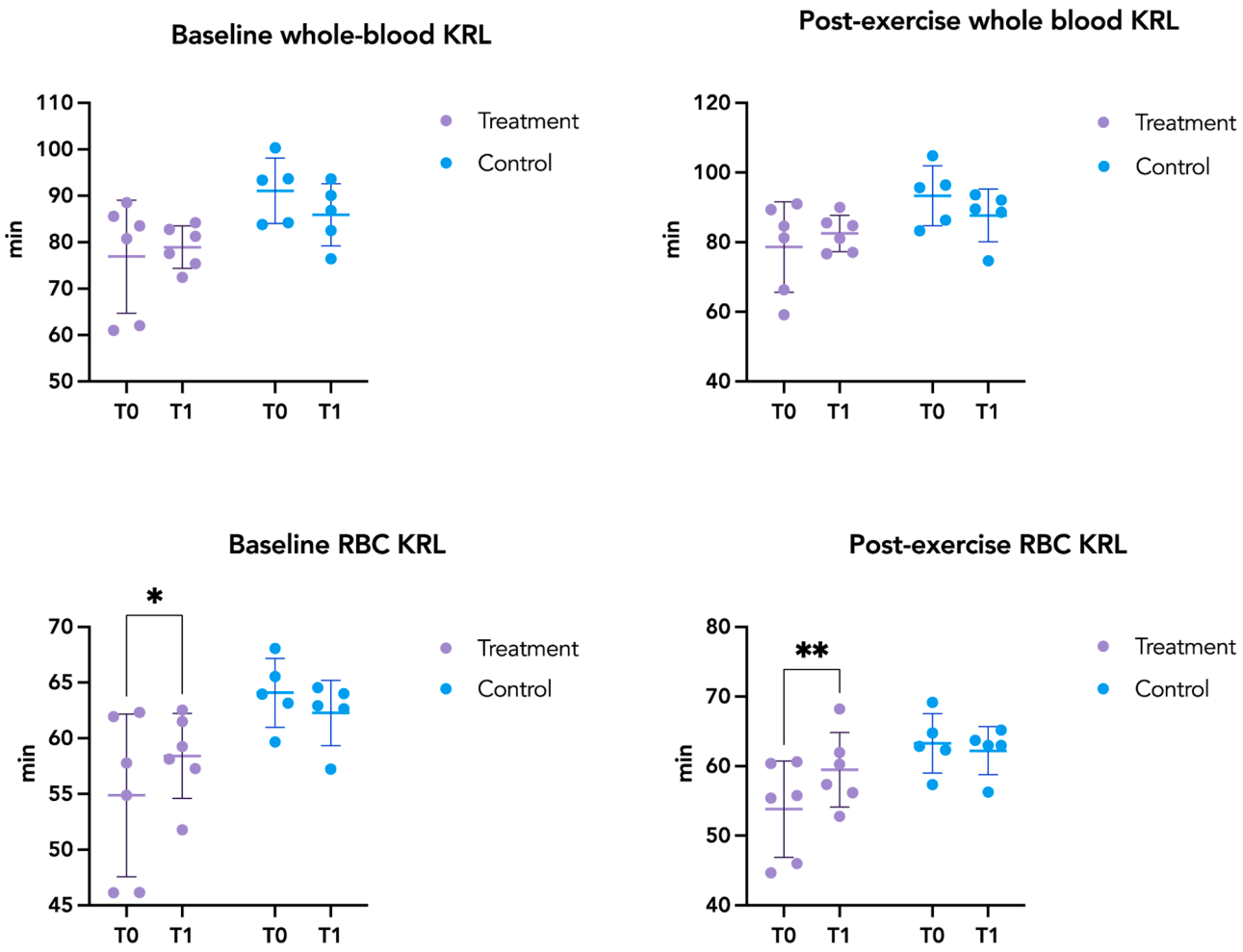


**Fig. 1.** Separated scatter plots showing the means and standard deviations of baseline and post-exercise whole-blood and red blood cell Kit Radicaux Libres (RBC KRL) test values in the treatment and control groups, at T0 and T1. Significant differences between T0 and T1 values are displayed as * ($P < 0.05$) and ** ($P < 0.01$).

omega-3 acids may modulate circulating triglyceride concentrations [53,54], preventing the activation of pro-inflammatory pathways and promoting the production of anti-inflammatory mediators [55,56]. Extensive investigations on the effects of DHA and EPA on the performance of human and equine athletes have shown promising results, demonstrating their roles in muscle remodeling and repair [57,58], reduce muscle soreness [59–62], and enhance training adaptation [40, 63] in people. In our study, a similar protective effect on skeletal muscle tissues can be argued, as evidenced by a decrease in exercise-induced muscular membrane leakage in horses receiving the supplement, leading to a significant reduction of post-exercise serum CK concentration. However, in a previous study on horses, the CK levels were not influenced by EPA and DHA supplementation [53]. This difference may be related to the composition of the dietary supplement used in the present study, that contains not only omega-3 fatty acids, but also antioxidant molecules, which may have acted synergistically.

Previous studies also reported the effects of dietary EPA and DHA on the fitness of horses, including reduced exercise-induced blood lactate increase [33], lower heart rate during exercise [53], decreased post-exercise pro-inflammatory cytokines [34], promotion of pH homeostasis during exercise [64], and overall performance improvement [65]. Nevertheless, in our study the dietary supplement did not affect the mean and maximum heart rates measured during exercise, the heart rates during recovery or the blood lactate concentration. Similarly, other studies involving standardized exercise tests in eventing horses [30] and racehorses [53], failed to detect any influence of omega-3 supplementation on performance variables, such as heart rate, lactate concentration and speed. The discrepancies in results may be explained by the different type of horses and exercise tests performed in different studies and in the dosage and composition of the dietary supplement. In our study, endurance horses primarily underwent aerobic exercise, and blood lactate concentrations at 30 min post-exercise were consistently below the aerobic-anaerobic threshold values of 4 mmol/L. In the same way, in the study on show jumpers, mean post-exercise blood lactate concentrations were below or slightly above the threshold, suggesting a predominantly aerobic exercise [33]. This is in contrast with the studies on eventing horses and racehorses, where more intense exercise tests led to lactate peaks well above the threshold, indicating a significant contribution from anaerobic metabolism [30,53].

The KRL values of whole blood and RBC are in line with the data previously reported in trotter horses during a training period [51]. Interestingly, a significant improvement was observed in the RBC antioxidant capacity. This result may be attributed to the tropism of omega-3 fatty acids for cellular membranes. DHA and EPA are incorporated into cellular membranes, modifying their structure and function [32,34,37,41]. They have also demonstrated efficacy in reducing exercise-induced osmotic fragility of RBC [33] and improving the fluidity and deformability of RBC membranes [53]. It should be noted that RBC antioxidant capacity of treated group increased both at rest and after exercise. A previous study demonstrated that pre-existing low-grade oxidative stress at baseline is associated with poor performance in endurance horses [20,23]. Given that endurance horses have a higher oxidant/antioxidant imbalance at rest compared to other sport horses [16], the improvement of their antioxidant capacity even before exercise

assumes relevance for their athletic condition and performance enhancement. To interpret our results correctly, it is important to consider that the supplement contains several active principles. Indeed, in addition to microalgae extract, the supplement included several plant extracts (*Vitis vinifera, Spirea ulmaria, Filipendula ulmaria*) containing polyphenols and vitamin C, providing an important source of antioxidants. *Vitis vinifera*, commonly known as grape, is abundant in polyphenols, including flavonoids, phenolic acids, tannins, stilbenes, and coumarins, which exert antioxidant, anti-inflammatory and cardiovascular protective activities [9,10,43,45]. Human athletes receiving grape extract supplementation have shown benefits in promoting oxidant/antioxidant status, reducing post-exercise CK, and enhancing physical performance [9]. However, a study exploring the effects of grape extract supplementation in horses did not reveal any significant impact on lactate nor CK concentrations [66]. Overall, polyphenols from several sources have demonstrated their ability to prevent exercise-induced oxidative stress, promote recovery, protect the muscular system, and improve cardiovascular function and aerobic capacity in humans [9,11,12,44]. The polyphenols are also present in *Spirea ulmaria* and *Filipendula ulmaria*, two plants belonging to the Rosaceae family, containing several flavonoids, phenolic acids, and tannins, recognized for their anti-inflammatory and antioxidant properties in human medicine [29,46–50]. To the best of the authors' knowledge, no studies have been published on effects of these plants on athletic performance or their use in equine species. In contrast, many studies have explored the role of vitamin C in sports performance in both humans and horses. Vitamin C, the most abundant hydrophilic antioxidant sub-stance, acts by neutralizing free radicals and potentiating the activity of vitamin E [7,25]. Human studies have reported contrasting results on the effects of vitamin C supplementation on athletic performance, with outcomes associated with the administered dosage [67]. Reported beneficial effects include the reduction of muscle soreness and CK levels, and the improvement of aerobic capacity [12]. In horses, vitamin C supplementation did not influence CK concentrations in endurance horses undergoing a 160 km race [15], while it improved antioxidant status and reduced muscle membrane leakage in another study on endurance horses after an 80 km race when administered in combination with vitamin E [25]. The different results observed in previous studies suggest that the effects of antioxidant supplementation on athletic performance can be related to several factors, including the type, intensity and duration of exercise, the dosage and length of dietary treatment, and the potential synergistic effects of mixing several compounds. In our study, the protective effects on skeletal muscle tissues likely could be the result from the interaction among several antioxidant molecules and anti-inflammatory omega-3 fatty acids, which seemed to be efficiently balanced and administered for a sufficient duration, and this was confirmed by the significant enhancement of antioxidant capacity in horses that received the dietary supplement.

Among the limitations of our study, it is essential to highlight the small sample size and the detected differences in whole-blood and RBC KRL values between groups at T0. However, the time*group interaction analyzed though two-way ANOVA should have helped mitigate this bias. It should be also observed that, in the present study, CK concentration was not measured at rest, and consequently the effect of the supplement on the muscle cannot be considered certain. Moreover, we evaluated the efficacy of a 21-day administration of the nutraceutical supplement at the dosage of 100 gr daily. However, future research is needed to test whether a longer duration of dietary supplementation or higher doses might produce even better effects or have detrimental effects. Indeed, studies in human athletes have revealed adverse effects of antioxidant or omega-3 fatty acids supplemented at dosages exceeding those recommended [12,37], mainly impeding skeletal muscle adaptation to endurance training [9,10]. Furthermore, it would be also of interest to evaluate the effect of the supplementation on Body Condition Score.

Finally, our study involved horses exercising for a relatively short distance (20 km) at a speed of 20 km/h. Previous studies have mainly focused on horses after 40 to 160 km endurance competitions, with mean speeds ranging from 8 to 16 km/h, and analyzed different oxidative stress biomarkers with different results [15,17,20–22,25,68–70]. Therefore, further investigations of the effects of the present nutraceutical supplement on the oxidative status of horses undergoing longer endurance rides are warranted. Similarly, due to the prevalent involvement of be-ta-oxidation of fatty acids in endurance exercise, our results may not be directly applicable to other equestrian disciplines characterized by high demands of anaerobic metabolism.

## 5. Conclusion

In conclusion, the 21-day administration of the nutraceutical supplement containing omega 3 fatty acids, plant antioxidants and vitamin C showed effectiveness in improving the total antioxidant capacity of RBC and reducing exercise-induced muscular damage in fit endurance horses. These results may be due to the synergistic action of the antioxidant and anti-inflammatory natural components of the supplement. Its inclusion in the diet of horses may be helpful for those engaged in intense aerobic exercise that involves a significant level of stress on the skeletal muscle system. Future studies should be undertaken to assess its effects on sport horses undergoing different types of training.

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**Data statement**

The data presented in this study are available upon reasonable request from the corresponding author.

**Ethic statement**

Ethical approval for the study was granted by the Animal Welfare Organization of the University of Milan (Protocol Number OPBA_16_2022). Written informed consent for the inclusion of patients in the present study was obtained from horse owners and/or delegated holders.

## CRediT authorship contribution statement

**L. Stucchi:** Writing – review & editing, Writing – original draft, Investigation, Data curation, Conceptualization. **R. Rossi:** Writing – review & editing, Formal analysis, Data curation. **E. Mainardi:** Writing – review & editing, Formal analysis, Data curation. **F. Ferrucci:** Writing – review & editing, Supervision, Investigation, Funding acquisition, Conceptualization.

## Declaration of competing interest

None of the authors has any financial or personal relationships that could inappropriately influence or bias the content of the paper.

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## References

[1] Zuffa T, Bennet ED, Parkin TDH. Factors associated with completion of Federation Equestre Internationale endurance rides (2012–2019): modelling success to

promote welfare-oriented decisions in the equestrian sport of endurance. Prev Vet Med 2022;198:105534.
[2] Ertelt A, Merle R, Stumpff F, Bollinger L, Liertz S, Weber C, et al. Evaluation of different blood parameters from endurance horses competing at 160 km. J Equine Vet Sci 2021;104:103687.
[3] Arfuso F, Giannetto C, Interlandi C, Giudice E, Bruschetta A, Panzera MF, et al. Dynamic metabolic response, clotting times and peripheral indices of Central fatigue in horse competing in a 44 km endurance race. J Equine Vet Sci 2021;106:103753.
[4] Fraipont A, Van Erck E, Ramery E, Richard E, Denoix JM, Lekeux P, et al. Subclinical diseases underlying poor performance in endurance horses: diagnostic methods and predictive tests. Vet Rec 2011;169:1545.
[5] De Moffarts B, Kirschvink N, Art T, Pincemail J, Lekeux P. Effect of oral antioxidant supplementation on blood antioxidant status in trained thoroughbred horses. Vet J 2005;169:65–74.
[6] Williams CA, Carlucci SA. Oral vitamin E supplementation on oxidative stress, vitamin and antioxidant status in in-tensely exercised horses. Equine Vet J Suppl 2006;36:617–21.
[7] Garcia EIC, Elghandour MMMY, Khusro A, Alcala-Canto Y, Tirado-Gonzàlez DN, Barbabosa-Pilego A, et al. Dietary supplements of vitamins E, C, and β-carotene to reduce oxidative stress in horses: an overview. J Equine Vet Sci 2022;110:103863.
[8] Urso ML, Clarkson PM. Oxidative stress, exercise, and antioxidant supplementation. Toxicology 2003;189:41–54.
[9] Elejalde E, Villaràn MC, Alonso RM. Grape polyphenols supplementation for exercise-induced oxidative stress. J Int Soc Sports Nutr 2021;18:3.
[10] Kruk J, Aboul-Enein BH, Duchnik E, Marchlewicz M. Antioxidative properties of phenolic compounds and their effect on oxidative stress induced by severe physical exercise. J Physiol Sci 2022;72(1):19.
[11] Bojarczuk A, Dzitkowska-Zabielska M. Polyphenol supplementation and antioxidant status in athletes: A narrative review. Nutrients 2023;15(1):158.
[12] Clemente-Suarez VJ, Bustamante-Sanchez A, Mielgo-Ayuso J, Martinez-Guardado I, Martìn-Rodrìguez A. Tornero-Aguilera J. Antioxidants and sports performance. Nutrients 2023;15(10):2371.
[13] Mills PC, Smith NC, Casas I, Harris P, Harris RC, Marlin DJ. Effects of exercise intensity and environmental stress on indices of oxidative stress and iron homeostasis during exercise in the horse. Eur J Appl Physiol 1996;74:60–6.
[14] Chiaradia E, Avellini L, Rueca F, Spaterna A, Porciello D, Antonioni MT, et al. Physical exercise, oxidative stress and muscle damage in racehorses. Comp Biochem Physiol B 1998;119:833–6.
[15] Hargreaves BJ, Kronfeld DS, Waldron JN, Lopes MA, Gay LS, Saker KE, et al. Antioxidant status and muscle cell leakage during endurance exercise. Equine Vet J Suppl 2002;34:116–21.
[16] Kinnunen S, Atalay M, Hyyppa S, Lehmuskero A, Hanninen O, Oksala N. Effects of prolonged exercise on oxidative stress and antioxidant defense in endurance horse. J Sports Sci Med 2005;4:415–21.
[17] Art T, Franck T, Gangl M, Votion D, Kohnen S, Deby-Dupont G, et al. Plasma concentrations of myeloperoxidase in endurance and 3-day event horses after a competition. Equine Vet J Suppl 2006;36:298–302.
[18] Smarsh DN, Liburt N, Streltsova J, McKeever K, Williams CA. Oxidative stress and antioxidant status in intensely exercising horses administered nutraceutical extracts. Equine Vet J 2010;42:317–22.
[19] Haggett E, Magdesian KG, Maas J, Puschner B, Higgins J, Fiack C. Whole blood selenium concentrations in endurance horses. Vet J 2010;186:192–6.
[20] Holbrook TC, McFarlane D, Schott II C. Neuroendocrine and non-neuroendocrine markers of inflammation associated with performance in endurance horses. Equine Vet J 2010;42:123–8.
[21] Bottegaro NB, Gotic J, Suran J, Brozic D, Klobucar K, Bojanic K, Vrbanac Z. Effect of prolonged submaximal exercise on serum oxidative stress biomarkers (d-ROMs, MDA, BAP) and oxidative stress index in endurance horses. BMC Vet Res 2018;14:216.
[22] Le Moyec L, Robert C, Triba MN, Bouchemal N, Mach N, Riviere J, et al. A first step toward unraveling the energy metabolism in endurance horses: comparison of plasma nuclear magnetic resonance metabolomic profiles before and after different endurance race distances. Front Mol Biosci 2019;6:45.
[23] Bollinger L, Bartel A, Weber C, Gehlen H. Pre-ride biomarkers and endurance horse welfare: analyzing the impact of the elimination of superoxide dismutase, δ-aminolevulinic-dehydratase, thiobarbituric acid reactive substances, iron, and serum amyloid A levels in elite 160 km endurance rides. Animals 2023;13(10):1670.
[24] Davis MS, Bayly WM, Hansen CM, Barrett MR, Blake CA. Effects of hyperthermia and acidosis on mitochondrial production of reactive oxygen species. Am J Physiol Regul Integr Comp Physiol 2023;325(6):725–34.
[25] Williams CA, Kronfeld DS, Hess TM, Saker KE, Waldron JN, Crandell KM, et al. Anti-oxidant supplementation and subsequent oxidative stress of horses during an 80-km endurance race. J Anim Sci 2004;82:594. 588.
[26] Robson P. Elucidating the unexplained underperformance syndrome in endurance athletes: the interleukin-6 hypothesis. Sports Med 2003;33:771–81.
[27] Serteyn D, Sandersen C, Lejeune JP, De La Rebiere, De Pouyade G, Ceuster J, Mouithys-Mickalad A, et al. Effect of a 120 km endurance race on plasma and muscular neutrophil elastase and myeloperoxidase concentrations in horses. Equine Vet J 2010;42:275–9.
[28] Williams CA, Burk AO. Nutrient intake during an elite level three-day event competition is correlated to inflammatory markers and antioxidant status. Equine Vet J 2010;42:116–22.
[29] Krstic M, Jovicic N, Selakovic D, Krstic B, Arsenijecvic N, Vasiliejevic M, et al. Simultaneous administration of hyperbaric oxygen therapy and anti-oxidant supplementation with Filipendula ulmaria extract in the treatment of thermal skin injuries alters nociceptive signalling and wound healing. Medicina 2023;59(9):1676.
[30] De Moffarts B, Portier K, Kirschvink N, Coudert J, Fellmann N, Van Erck E, et al. Effects of exercise and oral antioxidant supplementation enriched in (n-3) fatty acids on blood oxidant markers and erythrocyte membrane fluidity in horses. Vet J 2007;174(1):113–21.
[31] Kirschvink N, De Moffarts B, Farnir F, Pincemail J, Lekeux P. Investigation of blood oxidant/antioxidant markers in healthy competition horses of different breeds. Equine Vet J Suppl 2006;36:239–44.
[32] Portier K, De Moffarts B, Fellman N, Kirschvink N, Motta C, Letellierw C. The effects of dietary N-3 and antioxidant supplementation on erythrocyte membrane fatty acid composition and fluidity in exercising horses. Equine Vet J Suppl 2006;36:279–84.
[33] Bazzano M, Rizzo M, Arfuso F, Giannetto C, Fazio F, Piccione G. Increase in erythrocyte osmotic resistance following polyunsaturated fatty acids (PUFA) supplementation in show jumper horses. Livest Sci 2015;181:236–41.
[34] Hess T, Braun S, Herkelman K. The effects of various levels of docosahexaenoic acid on inflammatory markers in conditioned horses during lactate threshold tests. J Equine Vet Sci 2019;72:64–71.
[35] Adah AS, Ayo JO, Adah DA, Nwonuma CO, Lawal TA. Molecular docking and experimental validation of the effect of ergothioneine on heat shock protein-70 following endurance exercise by Arabian stallions. BMC Vet Res 2023;19(1):27.
[36] Williams CA. The effect of oxidative stress during exercise in the horse. J Anim Sci 2016;94:4067–75.
[37] Sembratowicz I, Zieba G, Cholewinska E, Czech A. Effect of dietary flaxseed oil supplementation on the redox status, haematological and biochemical parameters of horses' Blood. Animals 2020;10(12):2244.
[38] Saini RK, Prasad P, Sreedhar RV, Naidu KA, Shang X, Keum YS. Omega−3 polyunsaturated fatty acids (PUFAs): emerging plant and microbial sources, oxidative stability, bioavailability, and health benefits—A review. Antioxidants 2021;10(10):1627.
[39] Nyquist NF, Burri L, Jensen RB. Effect of dietary krill oil supplementation on horse red blood cell membrane fatty acid composition and blood parameters. J Anim Physiol Anim Nutr 2023;107:1251–61.
[40] Tomczyk M, Jost Z, Chroboczek M, Urbansky R, Calder PC, Fisk HL, et al. Effects of 12 weeks of omega-3 fatty acid supplementation in long-distance runners. Med Sci Sports Exerc 2023;55(2):216–24.
[41] Hess TM, Rexford JK, Hansen DK, Harris M, Schauermann N, Ross T, et al. Effects of two different dietary sources of long chain omega-3, highly unsaturated fatty acids on incorporation into the plasma, red blood cell, and skeletal muscle in horses. J Anim Sci 2012;90(9):3023–31.
[42] Elzinga SE, Betancourt A, Stewart JC, Altman MH, Barker VD, Muholland M, et al. Effects of docosahexaenoic acid rich microalgae supplementation on metabolic and inflammatory parameters in horses with equine metabolic syndrome. J Equine Vet Sci 2019;83:102811.
[43] Ghalishourani SS, Farzollahpour F, Shirinbakhshmasoleh M, Kolahndouz S, Ghaedi E, Behrouzian M, et al. Effects of grape products on inflammation and oxidative stress: A systematic review and meta-analysis of randomized controlled trials. Phytoter Res 2021;35(9):4898–912.
[44] Farhan M, Rizvi A. The pharmacological properties of red grape polyphenol resveratrol: clinical trials and obstacles in drug development. Nutrients 2023;15:4486.
[45] Di Pietro Fernandes C, Santana LF, Dos Santos JR, Fernandes DS, Hiane PA, Pott A, et al. Nutraceutical potential of grape (Vitis vinifera L.) seed oil in oxidative stress, inflammation, obesity and metabolic alterations. Molecules 2023;28(23):7811.
[46] Samardzic S, Arsenijevic J, Bozic D, Milenkovic M, Tesevic V, Maksimovic Z. Antioxidant, anti-inflammatory and gastroprotective activity of Filipendula ulmaria (L.) Maxim. And Filipendula vulgaris Moench. J Ethnopharmacol 2018;213:132–7.
[47] Kostikova VA, Petrova NV. Phytoconstituents and Bioactivity of plants of the genus spiraea L. (Rosaceae): a review. Mol Sci 2021;22:11163.
[48] Sukhikh S, Ivanova S, Skrypnik L, Bakhtiyarova A, Larina V, Krol O, et al. Study of the antioxidant properties of Filipendula ulmaria and Alnus glutinosa. Plants 2022;11(18):2415.
[49] Mihailovic V, Sreckovic N, Nedic ZP, Dimitrijevic S, Matic M, Obradovic A, et al. Green synthesis of silver nanoparticles using Salvia verticillata and Filipendula ulmaria extracts: optimization of synthesis, biological activities, and catalytic properties. Molecules 2023;28(2):808.
[50] Savina T, Lisun V, Feduraev P, Skrypnik L. Variation in phenolic compounds, antioxidant and antibacterial activities of extracts from different plant organs of Meadowsweet (Filipendula ulmaria (L.) Maxim.). Molecules 2023;28(8):3512.
[51] Corino C, Prost M, Pizzi B, Rossi R. Dietary plant extracts improve the antioxidant reserves in weaned piglets. Antioxidants 2021;10(5):702.
[52] Rossi R, Lo Feudo CM, Zucca E, Vizzarri F, Corino C, Ferrucci F. Innovative blood antioxidant test in standard-bred trotter horses. Antioxidants 2021;10(12):2013.
[53] O'Connor CI, Lawrence LM, Lawrence AC, Janicki KM, Warren LK, Hayes S. The effect of dietary fish oil supplementation on exercising horses. J Anim Sci 2004;82(10):2978–84.
[54] Wei MY, Jacobson TA. Effects of eicosapentaenoic acid versus docosahexaenoic acid on serum lipids: a systematic re-view and meta-analysis. Curr Atheroscler Rep 2011;13. 474e83.
[55] Gonzalez-Periz A, Horrillo R, Ferre N, Gronert K, Dong B, Moràn-Salvador E, et al. Obesity-induced insulin resistance and hepatic steatosis are alleviated by u-3 fatty acids: a role for resolvins and protectins. FASEB 2009;23(6):1946–57.

[56] Wall R, Ross RP, Fitzgerald GF, Santon C. Fatty acids from fish: the anti-inflammatory potential of long-chain omega-3 fatty acids. Nutr Rev 2010;68: 280–9.
[57] Deckelbaum RJ, Worgall TS, Seo T. N-3 fatty acids and gene expression. Am J Clin Nutr 2006;83:1520–5.
[58] Calder PC. N-3 PUFA and inflammation: from membrane to nucleus and from bench to bedside. Proc Nur Soc 2020;79:404–16.
[59] Lembke P, Capodice J, Hebert K, Swenson T. Influence of omega-3 (N3) index on performance and wellbeing in young adults after heavy eccentric exercise. J Sports Sci Med 2014;13(1):151–6.
[60] Harty PS, Cottet ML, Malloy JK, Kersksick CM. Nutritional and supplementation strategies to prevent and attenuate exercise-induced muscle damage: A brief review. Sports Med Open 2019;5:1.
[61] Ramos-Campo DJ, Avila-Gandia V, Lopez-Roman FJ, Minarro J, Contreras C, Soto-Mendez F, et al. Supplementation of re-esterified docosahexaenoic and eicosapentaenoic acids reduce inflammatory and muscle damage markers after exercise in endurance athletes: A randomized, controlled crossover trial. Nutr 2020;12(3):719.
[62] Kyriakidou Y, Wood C, Ferrier C, Dolci A, Elliott B. The effect of omega-3 polyunsaturated fatty acid supplementation on exercise-induced muscle damage. J Int Soc Sports Nutr 2021;18:9.
[63] Heileson JL, Machek SB, Harris DR, Tomek S, DeSouza LC, Kieffer AJ, et al. The effect of fish oil supplementation on resistance training-induced adaptations. J Int Soc Sports Nutr 2023;20(1):2174704.
[64] Dunnett CE, Marlin DJ, Harris RC. Effect of dietary lipid on response to exercise: relationship to metabolic adaptation. Equine Vet J Suppl 2002;34:75–80.
[65] Melo SKM, Diniz AIA, Lira VL, De Oliveira Muniz SK, GR Da Silva, Da CC Manso HEC, et al. Antioxidant and haematological biomarkers in different groups of horses supplemented with polyunsaturated oil and vitamin E. J Anim Physiol Anim Nutr 2016;100(5):852–9.
[66] Davies JA, Krebs GL, Barnes A, McGrath PJ. Feeding grape seed extract to horses: effects on health, intake and di-gestion. Animal 2009;3(3):380–4.
[67] Braakhuis A. Effect of vitamin C supplements on physical performance. Curr Sports Med Rep 2012;11:180–4.
[68] Lejeune JP, Sandersen C, Votion D, Caudron I, Vander Heyden L, Franck T, et al. Effect of intensive exercise on plasmatic neutrophil elastase level in eventing and endurance horses. Equine Vet J 2010;42(38):12–6.
[69] Mach N, Ramayo-Caldas Y, Clark A, Moroldo M, Robert C, Barrey E, et al. Understanding the response to endurance exercise using a systems biology approach: combining blood metabolomics, transcriptomics and miRNomics in horses. BMC Genom 2017;18:187.
[70] Marlin D, Fenn K, Smith N, Deaton CD, Roberts CA, Harris PA, et al. Changes in circulatory antioxidant status in horses during prolonged exercise. J Nutr 2002; 132:1622–7.