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Dietary antioxidant supplementation improves the *in vitro* quality and antioxidant capacity of Colombian Creole stallion semen

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Abstract: Dietary antioxidant supplementation has become popular for managing male infertility because of its impact on the antioxidant capacity of semen. This study evaluates the effect of dietary antioxidant supplementation on the seminal characteristics of Colombian Creole horses. Semen from six Colombian Creole stallions was collected using an artificial vagina. The stallions were randomly allocated to a control group (CG) or a supplementation group (SG) that received a multi-antioxidant supplement. A repeated measures design, with a wash out period of two weeks between treatments, was used. Sperm motility and kinematics were assessed using a computer-assisted semen analysis system, membrane integrity (MI) and the acrosomal integrity (AI) of sperm were assessed using fluorescent microscopy, abnormal morphology (AM) was analysed using supravital staining, and the functionality of the sperm membrane was assessed using the hypoosmotic swelling (HOS) test. A fraction of each ejaculate sample was centrifuged to obtain seminal plasma (SP). The reactive oxygen species and the total antioxidant capacity (TAC) of the SP were measured via spectrofluorimetry. Linear models were fitted, and comparison of means was performed using Tukey's test. Dietary antioxidant supplementation increased sperm beat cross frequency. In addition, a higher AI was found for the SG. Supplementation also increased the TAC of the SP. Individual analysis revealed that dietary antioxidant supplementation increased the total and progressive motility of the semen of two horses in the SG; however, supplementation reduced these two parameters in two other individuals compared to the CG. It can be concluded that dietary antioxidant supplementation with a nutraceutical that provides vitamins, minerals, amino acids, and omega-3 for horses can increase sperm quality as well as the TAC of SP; however, this may be subject to the individual effect of each horse.

Keywords: equine; nutraceutical; oxidative stress; sperm

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High-quality stallion semen is one of the most critical factors in a breeding programme; however, the cooling of semen for preservation for artificial insemination decreases motility, lifetime, fertility and viability of sperm, in addition to increasing the production of reactive oxygen species (ROS), all of which contribute to lipid peroxidation, changes in membrane permeability and deleterious effects on sperm quality. Furthermore, equine semen has a greater susceptibility to temperature change than other species, resulting in lower pregnancy rates when cooled semen is used for artificial insemination (Pena et al. 2019). Notwithstanding the importance of sperm quality, it is not uncommon to have aged or subfertile stallions with desirable genetics that produce poor-quality ejaculate containing a large number of spermatozoa with abnormalities, including low motility and low viability. For example, genetic traits seem particularly important when breeding Colombian Creole horses, which have been subjected to a particular set of selection practices that focus primarily on gait. Similarly, there is a preference for individuals with superior performance at equine fairs (Betancur et al. 2022). Therefore, stallion management and therapeutic treatments should be used to improve semen quality, facilitating the inclusion of stallions with valuable genetics but poor reproductive performance in breeding programmes (Stradaioli et al. 2004).

Over the last few years, nutritional supplements have been used to improve equine fertility (Bazzano et al. 2021), with dietary antioxidant supplementation becoming popular for the management of male infertility because of its impact on the antioxidant capacity of semen (Freitas and De Oliveira 2018). Horses cannot synthesise polyunsaturated fatty acids (PUFAs) such as omega-3, which they must acquire from PUFA precursors in their diet (Martin-Rosset et al. 2018). Omega-3 supports maintenance of the flexibility, elasticity and deformability of the sperm membrane, as well as membrane stability during cooling (Freitas and De Oliveira 2018). L-carnitine is another effective antioxidant for improving horse semen quality; it appears to play a role in the acquisition of sperm motility, modulates several metabolic functions, and correlates with high sperm concentration and progressive sperm motility (Stradaioli et al. 2004; Bazzano et al. 2021). Furthermore, L-carnitine improves the fertilising capacity of sperm, and oral supplementation with L-carnitine minimises lipid peroxidation during semen cooling (Stradaioli et al. 2004). It is well known that equine spermatozoa rely almost entirely on mitochondrial oxidative phosphorylation to meet their energy needs and generate ATP for motility, with a demonstrably poor capacity for glucose utilisation (Swegen et al. 2016). This mitochondrial activity predisposes stallion sperm to oxidative damage and high ROS production (Swegen et al. 2016). therefore, there is a significant risk of damage to DNA, proteins, lipids and membrane permeability (Pena et al. 2019). Although antioxidants cannot eliminate peroxidation entirely, they can delay ROS production and lessen oxidative stress linked to sperm damage (Bazzano et al. 2021).

Vitamins also play an important role in spermatogenesis and sperm viability. Vitamin E deficiency causes a high incidence of sperm abnormalities in equine sperm (Bazzano et al. 2021). Vitamin E and vitamin C have been reported to improve progressive motility, sperm concentration and morphology in human semen (Zhou et al. 2022). Furthermore, vitamin C deficiency impacts the androgen-sensitive parameters of the reproductive tissues of guinea pigs, altering sperm morphology and motility and decreasing fertility rates (Cheah et al. 2011).

The importance of amino acids in reproduction has also been established. For example, arginine, a nutritionally essential amino acid, plays a crucial role in spermatogenesis and sperm capacitation. Arginine is a biochemical precursor in the synthesis of putrescine, spermidine and spermine – molecules that are essential for sperm motility (Cheah et al. 2011). Studies on nutritional supplementation with amino acids such as lysine, methionine and threonine for boars have reported improvements in sperm concentration, motility parameters, acrosome integrity, morphologically normal spermatozoids and the number of live piglets at birth (Dong et al. 2016).

In addition, some microelements such as zinc have been found to be critical for the process of spermatogenesis (Usuga et al. 2017). The microelement, selenium, plays a vital role in testicular development, spermatogenesis, sperm motility and sperm viability (Cheah et al. 2011). Furthermore, low chrome concentrations in seminal plasma (SP) impact sperm production, motility and concentration in humans and animals (Bassey et al. 2013). Nutraceutical supplementation as a management strategy for improving semen quality opens up the possibility of using stallions with low fertility and

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desirable genetic traits in breeding programmes. Therefore, this study evaluates the effects of dietary antioxidant supplementation on the quality and antioxidant capacity of equine semen.

MATERIAL AND METHODS

This study received ethical endorsement for animal experimentation (Project No.: Ae-052, Act No. 10, Aug 2022) from the Ethics Committee of Universidad CES, Medellín, Colombia.

Experimental design

A repeated measures crossover design was used in this study, with six Colombian Creole horses, aged 5 to 15 years old, employed as subjects. The geographical site of the study was Rionegro, Antioquia, Colombia (6°09'12"N 75°22'27"W). The study was conducted between August and December 2022, and an average temperature of 16.4 °C, average daily minimum temperature of 13 °C and average daily maximum temperature of 23.2 °C were recorded; the average relative humidity was 84%. The temperature-humidity index (THI) values during the research period are presented in Figure 1.

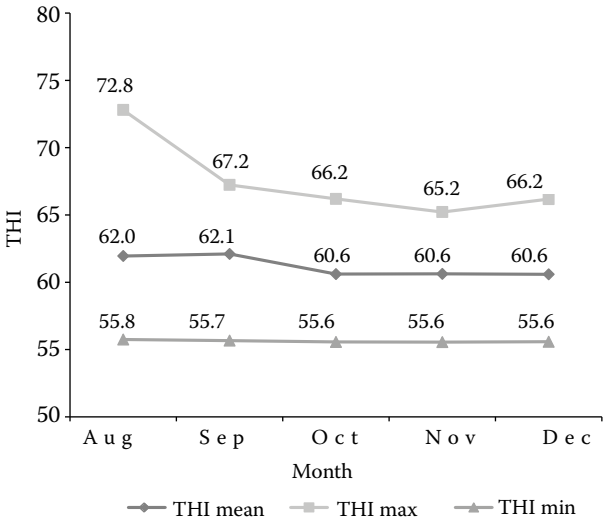

Figure 1. Temperature-humidity index (THI) for time period and the geographical area of the research

THI was calculated with the formula:

$THI = 0.8 \times T + RH \times (T - 14.4) + 46.4$

where:

T – the temperature;

RH – the relative humidity

THI max = temperature-humidity index with respect to the maximum temperature; THI mean = temperature-humidity index with respect to the average temperature; THI min = temperature-humidity index with respect to the minimum temperature.

The horses were housed in boxes and fed Pangola grass (*Digitaria eriantha*) hay *ad libitum*, along with water and 3 kg of grain and 30 g of mineralised salt per day. The nutritional composition of their standard diet is presented in Table 1. No nutritional supplements were given to the horses before the start of the study. The experiment in this study began with two weeks of adaptation, during which there was no supplementation. During this period, stallion semen was routinely collected three times a week. Subsequently, the stallions were randomly distributed into two groups: a control group (CG) that received no supplementation and a supplementation group (SG) that received a daily dose of 40 g of a nutritional supplement (Table 1) per animal administered with their morning feed. The stallions in the two study groups continued to receive their standard diet throughout the study period, with only the SG receiving the nutritional supplement. The horses remained in their assigned study group for eight weeks, during which semen quality was evaluated on two days each week. Once the first eight weeks were over, a wash out period of two weeks commenced. After the wash out period, the horses were switched to the opposite study group, such that the stallions in the CG were then supplemented for eight more weeks to complete the semen analysis. An eight-week supplementation period was chosen because a full spermatogenic cycle (spermatocytogenesis, meiosis and spermiogenesis) requires 57 days in these horses. The nutritional supplement described in Table 1 was selected for this study because of its composition, which comprises vitamins, fatty acids and antioxidant compounds that were not provided in the basal diet. In addition, some of the compounds in the nutritional supplement are considered essential, with the implication that horses cannot produce these compounds in sufficient quantities on their own and must obtain them from their diet (Bazzano et al. 2021).

Semen sample collection

Semen samples were collected from six Colombian Creole horses. As previously described, the horses

https://doi.org/10.17221/98/2024-CJAS

Table 1. Nutritional composition of the standard diet (hay, grain and mineralised salt) and the nutritional supplement

Pangola grass hay (*Digitaria eriantha*)	
Nutrient	quantity (%)
Dry matter	88
TDN	48.11
Protein	6.75
Calcium	0.32
Phosphorus	0.25
Fat	1.76
Fiber	33.93
Grain	
Nutrient	quantity (%)
Protein	15
Fat	3
Fiber	10
Ash	10
Moisture	13
Mineralised salt	
Nutrient	quantity (%)
Min calcium	16
Min phosphorus	6
Min salt	40
Max fluoride	0.06
Min magnesium	2
Min zinc	0.6
Min copper	0.1
Min iodine	0.002 7
Min cobalt	0.007
Min sulfur	1.5
Min selenium	0.001 5
Min moisture	5
Nutritional supplement (per kg of the product).	
Nutrient	quantity
Vitamin B12	900 µg
Vitamin B6	0.4 g
Vitamin A	916 666 IU
Vitamin E	40 000 IU
Vitamin C	100 g
Folic acid	20 g
Lysine	30 g
Methionine	16 g
Threonine	24 g
Arginine	10 g
L-carnitin	250 g
Omega 3	80 g
Zinc	2.8 g
Copper	1.1 g
Chrome	0.15 g
Selenium	0.2 g

max = maximum; min = minimum; IU = international units; TDN = t otal digestible nutrients

remained in one of the two study groups for eight weeks, during which semen quality was evaluated two days a week on the same days of the week (Wednesday and Friday) – although semen was routinely collected three times a week (every other day on business days) to lower the variability introduced by sexual rest. Semen collection was performed using a Missouri model artificial vagina lubricated with non-spermicidal gel, with assistance from a mare or a dummy. The gel fraction of the ejaculate was removed *via* filtration. The semen volume was evaluated using a graduated cylinder, sperm motility was analysed using light microscopy and sperm concentration was determined *via* spectrophotometry (SDM1; Minitube, Tiefenbach, Germany). Subsequently, the semen was diluted to a concentration of 100×10^6 cells per ml in a commercial equine semen extender, EquiPlus (Minitube, Tiefenbach, Germany), and then transported to the laboratory at 15 °C for further analysis.

Equine semen evaluation

In the laboratory, sperm motility and kinematics, sperm viability (SV), sperm morphology, the functionality of the sperm membrane and the acrosome reaction were evaluated.

Sperm motility and kinematics were assessed *via* computer-assisted semen analysis (CASA) using an integrated visual optical system (IVOS; Hamilton Thorne, Beverly, USA). For the CASA, 7 µl of semen was applied on a glass slide preheated to 37 °C and covered with a 20 × 20 mm coverslip. The setup also comprised negative phase optics, equine species, thermal plate at 37 °C and a particle size of 20–72 mm. A minimum of 500 spermatozoa were evaluated in a total of five observation fields. The parameters analysed were total motility (TM), progressive motility (PM), straight-line velocity (VSL), curvilinear velocity (VCL), average pathway

https://doi.org/10.17221/98/2024-CJAS

velocity (VAP), amplitude of lateral head displacement (ALH), beat cross frequency (BCF), straightness (STR), linearity (LIN) and rapid sperm (RAP).

To evaluate membrane integrity of sperm (MI), semen was diluted to a 20 × 10^6 spermatozoa/ml in Hanks Hepes (HH) medium with 1% bovine serum albumin (BSA) and incubated for 5 min at 35 °C with the nucleic acid dye SYBR14 (Thermo Fisher Scientific Inc., Waltham, USA) (to cause green fluorescence in sperm) to a final concentration of 6 mM. Afterwards, the spermatozoa were incubated in the same way but with nucleic acid fluorochrome propidium iodide (to cause red fluorescence in dead cells) at a concentration of 0.48 mM. Semen samples of 5 µl were placed on a glass slide with a 20 × 20 mm coverslip, and 200 cells were counted using a fluorescence microscope (T670Q-PL-FL; Amscope, Irvine, USA) at wavelengths of between 550 and 595 nm.

To determine the functionality of the sperm membrane, a modified hypoosmotic swelling (HOS) test was implemented. For the HOS procedure, 100 µl of semen was added to a tube with 500 µl of a 5.4% sucrose solution (100 mOsmol/l). This mixture was incubated at 38.5 °C for 30 min, and then the spermatic swelling of 200 cells was evaluated under at least five different power fields (400×) using an Eclipse E200 phase contrast microscope (Nikon Instruments Inc., Tokyo, Japan).

To evaluate sperm morphology, 200 sperm cells were analysed under a light microscope (1 000×) after semen staining using supravital staining and the eosin-nigrosin technique and were classified as morphologically normal or abnormal (AM). For this analysis, a slide was tempered at 37 °C, and a drop of semen was deposited at one end of the tempered slide, followed by a drop of eosin-nigrosin (Sigma-Aldrich, Saint Louis, USA). The two drops were then mixed for 30 to 60 s, extended onto the slide and fixed on a thermal plate at 37 °C.

To assess acrosomal integrity (AI), a FITC-PNA fluorescent probe (Sigma-Aldrich, Saint Louis, USA) was used. A sperm sample smear was made and fixed with 95% ethanol for 10 min. This was left to dry at room temperature and 25 µl of 5 µl/ml of FITC-PNA was added to phosphate-buffered saline solution (PBS). Each smear was kept in the dark for 30 min and washed with distilled water. Then, 200 sperm cells were evaluated using a fluorescence microscope (T670Q-PL-FL; Amscope, Irvine, USA).

Evaluation of the oxidative and antioxidant state of seminal plasma (SP)

Before dilution, a sample of 1 ml of each ejaculate was centrifuged (Sorvall ST 16; Thermo Fisher Scientific Inc., Waltham, USA) at 800 × *g* for 15 min to obtain the SP. Measurement of ROS was performed using the 2,7-dichlorodihydrofluorescein diacetate (H_2DCFDA) methodology. Each sample was prepared with 30 µl of SP, 240 µl of buffer solution (pH 7.4) and 30 µl of a 40 mM H_2DCFDA solution (Intervet International BV, Boxmeer, Holland). Controlled conditions were used (37 °C, pH 7.4) and the antioxidant Trolox (Merck, Darmstadt, Germany) was used as a reference. The readings were taken in quadruplicate using an LS 55 spectrofluorimeter (Perkin Elmer, Hopkinton, USA).

To determine the total antioxidant capacity (TAC) of the SP, a ferric-reducing ability of plasma (FRAP) assay was performed. For the FRAP assay, 50 µl of a sample was added to 900 µl of a FRAP solution composed of acid-sodium acetate buffer (pH 3.4), TPTZ and $FeCl_3$ in a 10 : 1 : 1 ratio. After 30 min of reaction, the absorbance was measured at a wavelength of 593 nm using a 6405 UV/Vis spectrophotometer (Jenway, Burlington, USA). The value obtained was compared with a reference curve constructed using ascorbic acid.

The TAC of each SP sample was also evaluated using the 2,2-azinobis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) test, for which 10 µl of SP and 990 µl of the radical $ABTS^{\bullet+}$ solution (Sigma-Aldrich, Saint Louis, USA) were used. After 30 min of reaction at room temperature in the dark, the change in absorbance was measured in triplicate in a 6405 UV/VIS spectrophotometer (Jenway, Burlington, USA), compared to a reference solution composed of 10 µl of a buffer solution and 990 µl of the radical $ABTS^{\bullet+}$ solution. The radical was generated via oxidation of 3.5 mM $ABTS^{\bullet+}$ with 1.25 mM potassium persulfate. After 24 h of reaction, the absorbance was adjusted with PBS at a pH of 7.4 up to 0.70 units at a lambda of 732 nm and compared against a standard curve with Trolox (Sigma Aldrich, Saint Louis, USA).

Statistical analysis

Data normality was assessed using the Shapiro–Wilk test, and the homogeneity of variances was verified using the Levene test. A linear mixed model

https://doi.org/10.17221/98/2024-CJAS

was fitted for each dependent variable to evaluate the sources of the variation. The nested effect of the ejaculate within the stallion, the fixed effects of the treatment and the date of sample collection were included in the adjusted models. The statistical model is as follows:

$$Y_{ijk} = \mu + T_{toi} + E_q\left(E_y\right)j + e_{ijk} \quad (1)$$

Where:

Y_{ijk} – sperm quality parameter;
μ – common mean for all treatments;
T_{toi} – fixed effect of treatment i (CG and SG);
$E_q(E_y)j$ – nested random effect of the interaction between stallion and ejaculate j;
e_{ijk} – random error.

Comparison of means was performed using Tukey's test. A significance level of P-value < 0.05 was established for all analyses, which were conducted using SAS Studio (v3.81; SAS Institute Inc., Cary, USA). The results are expressed as the mean ± standard error of the mean (SEM).

RESULTS

The results (mean ± SEM) for the semen quality of the samples collected in this study were as follows: a sperm concentration of 132.76 ± 5.62 × 10^6 cells/ml, TM of 73.5 ± 1.79%, PM of 34.9 ± 1.93%, SV of 69.78 ± 1.34%, HOS of 55.32 ± 1.18%, AI of 89.19 ± 0.57% and AM of 33.2 ± 1.12%.

Regarding sperm kinematics, it was found that the SG had sperm with a higher BCF than the sperm from the CG (P-value < 0.05, Table 2). Furthermore, the SG had sperm with a higher AI than the sperm from the CG, which did not receive nutritional supplementation (Figure 2, P-value < 0.05). The analysis of the oxidative-antioxidant state of the SP revealed higher FRAP in the supplemented animals than in the stallions in the CG, while for ABTS, higher values were recorded for the CG than the SG (P-value < 0.05; Table 3).

Individual comparisons between stallions showed greater changes in sperm motility and kinematics among the supplemented stallions compared to the stallions in the CG (Table 4). Three of the stallions

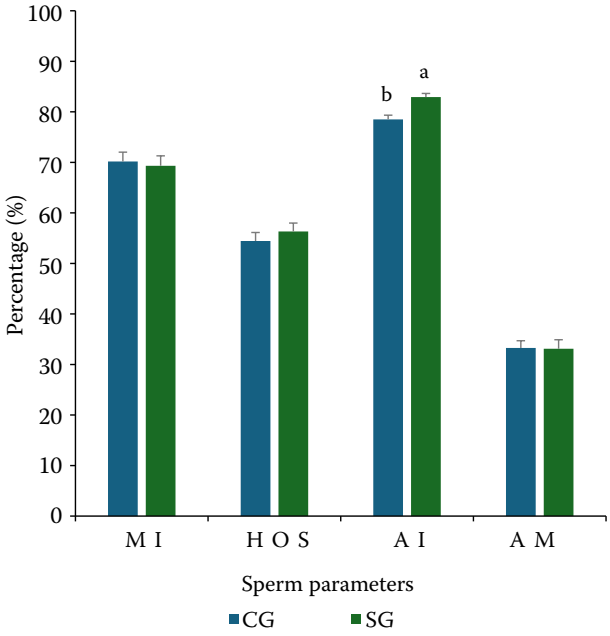

Figure 2. Results of equine sperm quality for the non-supplemented stallions (CG) and supplemented stallions (SG)
[a,b]Letters in the same bar indicate significant statistical difference between groups (P-value < 0.05); results are presented as mean ± standard error of the mean
AI = acrosomal integrity (%); AM = abnormal morphology (%); CG = control group (non-supplemented stallions); HOS = functionality of the sperm membrane (%); MI = membrane integrity of sperm (%); SG = supplemented group (supplemented stallions)

Table 2. Results of equine sperm motility and kinematics for the non-supplemented and supplemented stallions

Group	TM	PM	VAP	VSL	VCL	ALH	BCF	STR	LIN	RAP
CG	73.4 ± 2.5[a]	34.3 ± 2.4[a]	84.7 ± 2.6[a]	59.3 ± 2.4[a]	161.9 ± 4.4[a]	7.1 ± 0.2[a]	32.6 ± 0.7[b]	68.6 ± 1.2[a]	37.6 ± 1.0[a]	58.1 ± 2.8[a]
SG	73.5 ± 2.5[a]	35.6 ± 3.1[a]	88.9 ± 3.9[a]	62.4 ± 3.3[a]	167.7 ± 5.8[a]	6.8 ± 0.2[a]	36.6 ± 0.8[a]	68.6 ± 1.6[a]	37.5 ± 1.3[a]	58.6 ± 3.4[a]

[a,b]Letters in the same column indicate significant statistical difference between groups at P-value < 0.05; results are presented as mean ± standard error of the mean
ALH = lateral head displacement (μm); BCF = beat cross frequency (Hz); CG = control group (non-supplemented stallions); LIN = linearity (%); PM = progressive motility (%); RAP = rapid sperm (%); SG = supplemented group (supplemented stallions); STR = straightness (%); TM = total motility (%); VAP = average pathway velocity (μm/s); VCL = curvilinear velocity (μm/s); VSL = straight-line velocity (μm/s)

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Table 3. Analysis of the oxidative-antioxidant state of equine seminal plasma for the non-supplemented (CG) and supplemented (SG) stallion

Group	ROS	ABTS	FRAP
CG	0.042 ± 0.001[a]	5077.0 ± 179.2[a]	15.3 ± 0.9[b]
SG	0.041 ± 0.001[a]	4543.0 ± 186.0[b]	18.8 ± 1.7[a]

[ab]Different letters in the same column indicate significant statistical difference between groups (P-value < 0.05); results are presented as mean ± standard error of the mean
ABTS = µmol Trolox/L sample; CG = control group (non-supplemented stallions); FRAP = ferric reducing ability (equivalents mg of ascorbic acid/L sample); ROS = reactive oxygen species (relative fluorescence units – RFU); SG = supplemented group (supplemented stallions)

evaluated (equine 3, 4 and 5) showed an increase in most parameters of sperm motility and kinematics when they were supplemented (P-value < 0.05). For equine 6, no effect from nutritional supplementation was evident. In contrast. Equine 1 and equine 2 recorded decreases in TM, PM, STR, LIN and RAP when supplemented (P-value < 0.05). These observations demonstrate the individual effect of each stallion regarding their response to supplementation (Table 4).

The effect of nutritional supplementation on semen quality was also evaluated for each stallion individually, and we found that for equine 4, supplementation increased AI compared to the control: 84.00 ± 1.74% *vs* 74.16 ± 1.74%, respectively (P-value < 0.05). However, for this same horse (equine 4), supplementation reduced TAC, as assessed using the ABTS test, compared to the control: 354 3 ± 115.49 µmol Trolox/l sample *vs* 5 725.57 ± 115.49 µmol Trolox/l sample, respectively (P-value < 0.05). Conversely, when supplemented, equine 8 recorded an increase in TAC, as evaluated using the FRAP test, compared to the control: 35.46 ± 3.89 mg of ascorbic acid/l sample *vs* 25.29 ± 2.75 mg of ascorbic acid/l sample (P-value < 0.05).

The correlation analysis revealed a positive relationship between ROS and LIN in the CG (P-value < 0.05) and a positive relationship between ROS and SV in the SG (P-value < 0.05). Similarly, TAC, as evaluated using the ABTS assay, revealed a positive relationship (P-value < 0.05) between RAP and semen quality parameters such as TM and PM for the SG; however, a negative relationship (P-value < 0.05) was found between RAP and parameters such as TM, PM, VSL, ALH, STR and LIN for the CG (Table 5, Figure 3).

Table 4. Effect of nutritional supplementation on results of sperm motility and kinematics for each horse

	Equine													
	1		2		3		4		5		6			
	CG	SG	CG	SG	CG	SG	CG	SG	CG	SG	CG	SG		
TM	83.1 ± 4.86[a]	57.0 ± 5.13[b]	88.1 ± 5.28[a]	67.5 ± 5.91[b]	60.2 ± 5.65[b]	78.5 ± 5.65[a]	53.5 ± 6.18[a]	65.4 ± 6.18[a]	80.6 ± 2.44[b]	89.8 ± 2.44[a]	78.9 ± 4.85[a]	80.1 ± 6.86[a]		
PM	42.6 ± 4.37[a]	18.6 ± 4.61[b]	54.5 ± 5.41[a]	22.8 ± 6.05[b]	24.5 ± 6.03[a]	33.4 ± 6.03[a]	12.5 ± 4.90[b]	32.5 ± 4.90[a]	51.4 ± 2.78[b]	66.0 ± 2.78[a]	25.0 ± 3.57[a]	28.1 ± 5.05[a]		
VAP	78.7 ± 7.04[a]	67.8 ± 7.42[a]	101.0 ± 7.24[a]	59.3 ± 8.10[b]	81.8 ± 7.28[b]	108.4 ± 7.28[a]	68.5 ± 7.30[a]	87.6 ± 7.30[a]	90.2 ± 5.35[b]	110.8 ± 5.35[a]	90.0 ± 3.92[a]	80.0 ± 5.55[a]		
VSL	59.2 ± 5.57[a]	45.4 ± 5.87[a]	81.6 ± 6.57[a]	40.5 ± 7.35[b]	57.6 ± 6.30[a]	67.9 ± 6.30[a]	39.8 ± 5.59[b]	62.3 ± 5.59[a]	70.8 ± 3.88[b]	90.3 ± 3.88[a]	50.7 ± 2.78[a]	50.5 ± 3.94[a]		
VCL	163.5 ± 13.2[a]	142.1 ± 14.1[a]	179.2 ± 11.4[a]	121.1 ± 12.8[b]	151.3 ± 12.0[b]	198.9 ± 12.0[a]	139.4 ± 11.9[a]	173.6 ± 11.9[a]	165.4 ± 7.88[a]	183.9 ± 7.88[a]	175.9 ± 7.07[a]	160.8 ± 0.01[a]		
ALH	8.51 ± 0.39[a]	8.07 ± 0.41[a]	7.14 ± 0.63[a]	6.75 ± 0.71[a]	6.40 ± 0.46[a]	6.95 ± 0.46[a]	7.99 ± 0.36[a]	6.57 ± 0.36[b]	6.92 ± 0.28[a]	6.04 ± 0.28[b]	6.25 ± 0.54[a]	7.33 ± 0.77[a]		
BCF	29.4 ± 1.46[a]	31.2 ± 1.54[a]	36.6 ± 1.99[a]	31.9 ± 2.29[a]	31.8 ± 1.82[b]	39.1 ± 1.82[a]	31.0 ± 1.71[b]	39.0 ± 1.71[a]	34.3 ± 1.23[b]	42.9 ± 1.23[a]	32.8 ± 1.41[a]	28.9 ± 2.00[a]		
STR	73.7 ± 2.12[a]	64.2 ± 2.24[b]	76.5 ± 3.34[a]	64.1 ± 3.74[b]	68.7 ± 3.52[a]	64.4 ± 3.52[a]	58.0 ± 2.72[b]	67.7 ± 2.72[a]	77.2 ± 1.15[b]	81.7 ± 1.15[a]	59.5 ± 2.15[a]	65.0 ± 3.04[a]		
LIN	36.4 ± 1.20[a]	31.5 ± 1.26[b]	45.1 ± 2.19[a]	32.5 ± 2.45[b]	40.0 ± 2.81[a]	36.4 ± 2.81[a]	30.3 ± 2.55[a]	35.6 ± 2.55[a]	43.2 ± 1.39[b]	50.5 ± 1.39[a]	31.7 ± 1.74[a]	33.3 ± 2.47[a]		
RAP	65.7 ± 6.65[a]	35.7 ± 7.01[b]	74.0 ± 6.62[a]	37.6 ± 7.40[b]	44.7 ± 6.42[b]	68.8 ± 6.42[a]	33.1 ± 6.80[a]	50.6 ± 6.80[a]	70.2 ± 3.03[b]	84.4 ± 3.03[a]	64.8 ± 4.83[a]	64.6 ± 6.83[a]		

[ab]Different letters in the same row in the same horse, indicate significant statistical difference (P-value < 0.05); results are presented as mean ± standard error of the mean
ALH = lateral head displacement (µm); BCF = beat cross frequency (Hz); CG = control group; LIN = linearity (%); STR: straightness (%); PM = progressive motility (%); RAP = rapid sperm (%); SG = supplemented group ;TM = total motility (%); VAP = average pathway velocity (µm/s); VCL = curvilinear velocity (µm/s); VSL = straight-line velocity (µm/s)

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Table 5. Correlation analysis between sperm quality and oxidant-antioxidant status of equine seminal plasma, for the non-supplemented stallions (CG) and supplemented stallions (SG)

	Group	TM	PM	VAP	VSL	VCL	ALH	BCF	STR	LIN	RAP	MI	HOS	AI	AM
ROS	CG	−0.08	−0.03	−0.08	−0.03	−0.08	−0.03	−0.08	−0.03	0.22*	−0.09	0.15	0.01	−0.12	0.19
	SG	0.21	0.22	0.16	0.19	0.09	−0.06	0.01	0.09	0.15	0.18	0.29*	−0.02	–	-0.30*
ABTS	CG	−0.28*	-0.49*	−0.12	−0.42*	−0.13	−0.27*	−0.14	−0.50*	-−0.29*	−0.25*	−0.01	−0.06	−0.002	0.12
	SG	0.27*	0.26*	0.12	0.19	0.12	-0.07	0.01	0.22	0.20	0.30*	-0.02	0.08	0.01	0.08
FRAP	CG	0.002	−0.36*	−0.07	−0.32*	−0.04	0.07	−0.22	−0.41*	−0.29*	−0.04	−0.01	−0.09	0.08	−0.10
	SG	0.17	−0.03	0.10	−0.009	0.13	0.03	−0.04	−0.13	−0.06	0.17	−0.006	0.06	0.04	0.10

*Indicate significant statistical difference (P-value < 0.05)

ABTS = µmol Trolox/l sample; ALH = lateral head displacement (µm); AM = abnormal morphology (%); BCF = beat cross frequency (Hz); CG = control group (non-supplemented stallions); FRAP = ferric reducing ability (equivalents mg of ascorbic acid/l sample) HOS = functionality of the sperm membrane (%); LIN = linearity (%); PM = progressive motility (%); RAP = rapid sperm (%); ROS = reactive oxygen species (RFU = relative fluorescence units); SG = supplemented group (supplemented stallions); STR = straightness (%); TM = total motility (%); VAP = average pathway velocity (µm/s); VCL = curvilinear velocity (µm/s); VSL = straight-line velocity (µm/s)

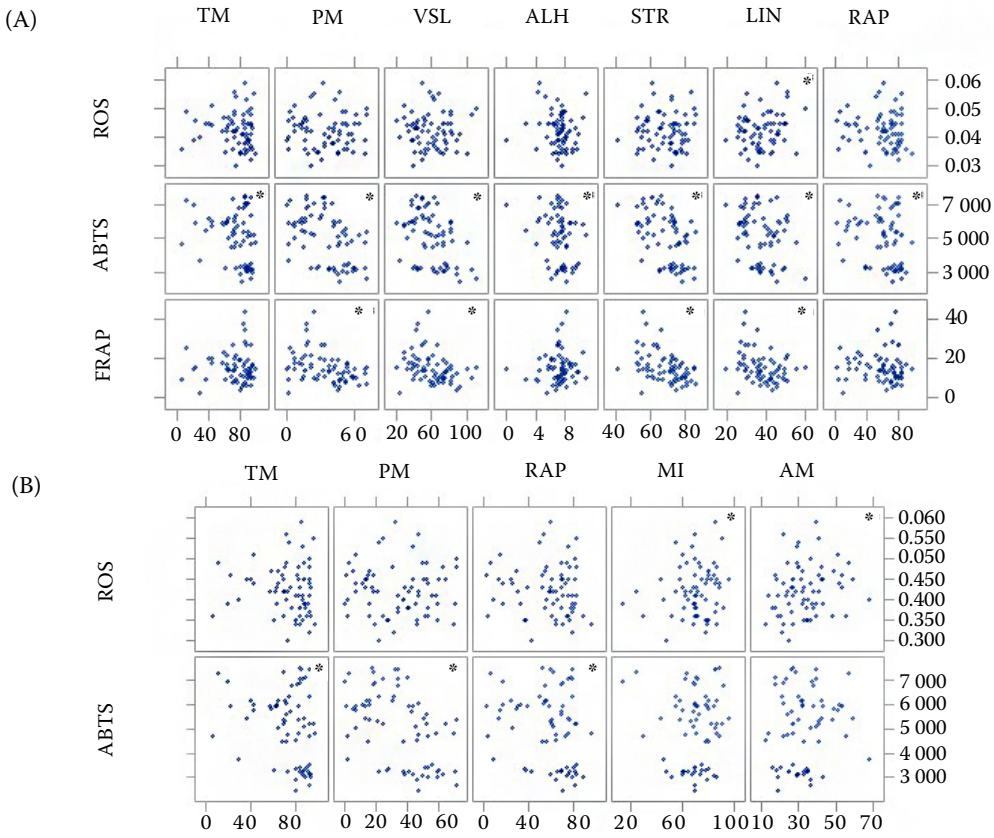

Figure 3. Correlation analysis plots between variables of sperm quality and oxidant-antioxidant status of equine seminal plasma, for the (A) non-supplemented stallions (CG) and (B) supplemented stallions (SG)

*Indicate significant correlation coefficients (P < 0.05); the dots in the correlation plot between each pair of dependent variables represent the repetitions of the measurements of sperm quality and oxidant-antioxidant status of equine seminal plasma; ABTS = µmol Trolox/l sample; ALH = lateral head displacement (µm); AM = abnormal morphology (%); BCF = beat cross frequency (Hz); CG = control group (non-supplemented stallions); FRAP = ferric reducing ability (equivalents mg of ascorbic acid/l sample) HOS = functionality of the sperm membrane (%); LIN = linearity (%); PM = progressive motility (%); RAP = rapid sperm (%); ROS = reactive oxygen species (RFU = relative fluorescence units); SG = supplemented group (supplemented stallions); STR = straightness (%); TM = total motility (%); VAP = average pathway velocity (µm/s); VCL = curvilinear velocity (µm/s); VSL = straight-line velocity (µm/s)

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DISCUSSION

Nutritional supplements are widely used in the equine industry with the aim of improving horse health and sports or reproductive performance. Over the years, several studies have focused on investigating the effects of several dietary compounds on the quality and preservation of stallion semen (Bazzano et al. 2021). The results of this study showed that dietary supplementation improves the BCF of sperm (Table 2), which is a useful indicator for evaluating the swimming pattern of sperm cells and can be linked to fertilisation rate (Kato et al. 2001). It has been proposed that sperm have a more progressive pattern of motility when they have smaller (lower ALH) but more frequent (higher BCF) head movements (Papin et al. 2021). Furthermore, BCF has been positively correlated with highly motile sperm subpopulations (King et al. 2000); however, some other studies have linked this parameter to sperm hyperactivation and DNA fragmentation (Aghazarian et al. 2021), while others have concluded that the sensitivity of BCF for detecting adverse effects on sperm motion is unclear (Kato et al. 2001). Although advanced, automated semen analysis methods such as CASA offer many advantages including standardisation, speed, accuracy and objectivity, their parameters do not reliably predict fertility in equines (Whitesell et al. 2020). Previous studies conducted by our research group on Colombian Creole horses show low levels of sperm DNA fragmentation, ranging from 0.02 ± 0.01 to 0.12 ± 0.04%, as assessed using flow cytometry (Restrepo et al. 2019). Thus, we did not prioritise this parameter in our semen quality assessment in this study. However, it would be interesting to evaluate not only DNA fragmentation but also the in vivo fertilising capacity of sperm in future studies.

Our results in this study also show that the supplemented stallions had a higher percentage of spermatozoa with AI (Figure 1). Some researchers have reported that acrosome damage or inability to perform acrosome reactions may impact the fertilisation capacity of sperm cells (Baldi et al. 2000). Acrosomes must be intact for the sperm to penetrate the oocyte. In addition, the intactness of the acrosome has been considered a critical factor for successful cryopreservation and sperm cell resistance to thermal and osmotic changes (Olexikova et al. 2019). Other studies have demonstrated that oral supplementation of stallions with antioxidants and fatty acids increases plasmatic and acrosomal membrane integrity, which may be due to the combined effect of high concentrations of PUFAs and the prevention of excessive lipid peroxidation by antioxidants (Freitas and de Oliveira 2018).

Dietary intervention can play a crucial role in replacing nutritional deficiencies that may affect sperm production and function, as well as antioxidant capacity (Bazzano et al. 2021). Supplemented stallions in this study recorded higher values for SP of TAC, as evaluated using the FRAP test, than the stallions in the CG (Table 3). The FRAP test is a simple assay for determining total antioxidant power based on the reduction of a ferric-tripyridyl triazine complex to its ferrous-coloured form in the presence of antioxidants (Tvrdai et al. 2016). Alterations in redox homeostasis have been acknowledged as a substantial trigger of infertility in both men and stallions (Pena et al. 2019). Spermatozoa normally produce ROS as a by-product of mitochondrial activity (Bazzano et al. 2021), and a balance between ROS production and antioxidants is critical for sperm function and survival. TAC evaluation is the analytical method most commonly used to determine the antioxidant balance in a biological sample (Catalan et al. 2022). A positive correlation has been reported between FRAP results and sperm motility in stallions (Tirpak et al. 2021); however, our results did not show any significant relationship between FRAP and seminal quality parameters in the SG. By contrast, a negative relationship was evident between the FRAP results and semen quality in the CG (Table 5). This might be linked to pro-oxidant molecules – molecules that function as antioxidants but, at improper concentrations, can turn pro-oxidant and harmful to sperm quality (Cheah et al. 2011; Hussain et al. 2023). The ABTS test results revealed positive correlations between semen quality parameters in the CG (Table 5), which showed higher results on this assay than the SG (Table 3). The ABTS test is a single-electron transfer-based assay, and its limitations are its pH dependency and reliance on the percentage of the product decrease instead of the kinematics (Bartosz 2010). It has been reported that when using a hydrophilic assay, such as the ABTS, most of the antioxidant capacity can be attributed to proteins, uric acid and ascorbic acid, while the effect of lipophilic components is minimal (Usuga et al. 2023). This could explain why oral supplementation with antioxidants, some of them lipophilic and fatty acids, did not produce an increase in this parameter.

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ROS production in stallion SP was not affected by oral supplementation (Table 3); however, there was a positive correlation between ROS and sperm LIN in the CG and SV in the SG (Table 5). ROS might not always be linked to detrimental effects on sperm (Hussain et al. 2023), although the physiological ROS levels are considered important for the proper accomplishment of different functions associated with gamete fertility, including proliferation, maturation, capacitation, hyperactivation, acrosomal reaction, fertilisation and the release of oocytes (Qamar et al. 2023). In our study, the relation between ROS and different semen quality parameters, may be due to considerable mitochondrial activity and ATP production by alive and morphologically normal spermatozoa (Barati et al. 2020).

In this study, the individual effect of each horse influenced the response to oral supplementation (Table 4). In other studies, it has been reported that not only seminal quality but also SP composition vary greatly from stallion to stallion (Usuga et al. 2017). These variations may also be affected by some environmental factors, such as temperature, nutrition, stress and the season during which the samples were collected (Tirpak et al. 2021) and may influence response to supplementation. Oral administration of L-carnitine may improve semen quality in stallions with poor fertility, although no effect has been observed in fertile stallions (Stradaioli et al. 2004). Furthermore, it has been reported that horses producing semen samples with good quality and freezability and therefore possibly high TAC should not need supplementation to increase antioxidant capacity, with supplementation having no significant effect or even a deleterious effect if administered in excess (Usuga et al. 2020). Furthermore, dietary antioxidants may generally require long-term and especially persistent treatment protocols to yield benefits in terms of male fertility (Qamar et al. 2023).

In this study, nutrients such as vitamins, L-carnitine, omega-3 and certain amino acids and ions were included in the horses' diet only through nutritional supplementation because their original standard diet lacked these compounds (Table 1). Horses, similar to other animals, are unable to self-synthetize PUFAs and some essential vitamins. The biosynthetic capacity of producing carnitine from lysine and methionine may be a limiting factor in horses, and, therefore, carnitine is often regarded as a conditionally essential nutrient (Bazzano et al. 2021). However, synergism and antagonism between dietary constituents are well-known phenomena; for example, in the presence of transition metals, vitamin C, considered an important antioxidant, makes radicals highly reactive and more destructive, thus generating more free radicals. In addition, vitamin C has the capacity to promote the release of these transition metals from proteins, which further contributes to this effect of generating free radicals (Vasconcelos et al. 2013). The findings of some studies even indicate that high levels of vitamin C in stallion SP are linked to low fertility and diminished seminal quality (Vasconcelos et al. 2013; Usuga et al. 2017). Similarly, a negative correlation has been reported between zinc levels and the vitality and MI of equine sperm (Usuga et al. 2017).

CONCLUSION

Diet supplementation of stallions with a nutraceutical that provides vitamins, minerals, amino acids, and omega-3 can increase the TAC of SP, as well as sperm quality; however, this may be subject to the differential and individual effect of each horse. Further studies are needed to evaluate the effect of nutraceutical supplementation not only on semen quality but also on in vivo fertility in equine species, as well as experimentation on larger animal samples to develop optimal supplementation strategies.

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Conflict of interest

The authors declare no conflict of interest.

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