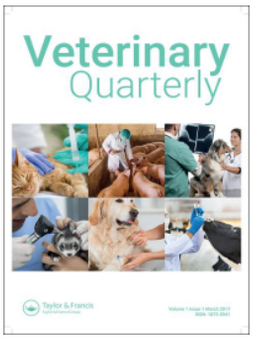

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Effects of oral black Maca (*Lepidium meyenii*) supplementation on semen quality and refrigerated storage stability in subfertile and normofertile dogs: a study on sperm parameters and testosterone levels

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ABSTRACT

The aim of this study was to assess the beneficial effects of oral supplementation of *Lepidium meyenii* (Maca) on improving and keeping sperm quality in dogs during storage, and to investigate its effect on changes in testosterone concentrations. Forty male dogs were enrolled in the study and divided into four groups of ten dogs each: two subfertile (control and treatment) and two normofertile (control and treatment) groups. The dogs in the treatment groups received Maca in a capsule formulation (75 mg/kg), while the control groups received placebo. The spermiogram and testosterone levels were assessed at three times of the sperm cycle: 0 (T0), 31 (T31), and 62 (T62) days. Ejaculates were processed for storage at 5 °C and evaluated for total and progressive motility and membrane integrity at 3 (T3h), 24 (T24h), 48 (T48h), and 72 h (T72h) post storage. The oral supplementation of 75 mg/kg of Maca extract in dogs can improve sperm parameters and increase serum testosterone concentrations, leading to improved reproductive capacity. The semen of subjects treated with oral Maca supplementation maintained its parameters stable for a longer period when stored compared to the semen of control subjects, demonstrating the beneficial effect of the use of this extract on male fertility.

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KEYWORDS
Maca; *Lepidium meyenii*; dietary supplement; canine cooled semen; testosterone; sperm parameters; reproduction

Introduction

The use of cooled semen for artificial insemination (AI) in dogs is significantly increasing (Goericke-Pesch et al. 2012; Hollinshead and Hanlon 2017). This trend is related to the reduced stress on the animal, as only the donor semen needs to be transported to the recipient bitch, and the greater logistical convenience of short-term semen storage. Compared to frozen semen, the use of cooled semen shows high efficiency in breeding management, resulting in improved pregnancy rates and a wider dissemination of canine genetics worldwide (Goericke-Pesch et al. 2012; Mason 2018). However, *in vitro* fertility of cooled sperm is only maintained for a maximum of 24-48 h (Camilo Hernández-Avilés 2021). This leads to reduced biologically acceptable outcomes when cooled semen is used for insemination beyond 48 h (Hesser et al. 2017; Hollinshead and Hanlon 2017; Camilo Hernández-Avilés 2021). Storage at refrigeration temperatures leads to a decrease in semen quality due to an increase in reactive oxygen species (ROS) and the accumulation of declining spermatozoa. This condition negatively affects fertility and pregnancy rates (Linde-Forsberg 1991; Cheuquemán et al. 2012; Kasimanickam et al. 2012; Tesi et al. 2018; Camilo Hernández-Avilés 2021).

Lipids, the major constituents of the sperm plasma membrane, regulate numerous biochemical and functional processes required for fertilisation. These processes include maturation, hyperactivation, capacitation, acrosome reaction (Alvarez and Storey 1995), sperm-oocyte binding and fusion (Martínez and Morros 1996; Flesch and Gadella 2000; Tavilani et al. 2005). During the process of spermatogenesis, and especially during the maturation phase that takes place in the epididymis, the sperm membrane undergoes an active lipid metabolism that leads to a continuous rearrangement of its lipid profile, characterised by the decrease of cholesterol and the desaturation

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Table 1. Age (years) of subjects included in the study.

Groups	Age of each subjects										Mean ± SD
Subfertile control	6	3,5	3	3	2	2,5	3	4	3	2	3,2 ± 1,159
Normofertile control	4	3	2,5	4,5	5	4,5	2,5	3	4	4,5	3,75 ± 0,92
Subfertile treatment	3	3,5	2	2	4	5	6	4,5	4	3	3,7 ± 1,273
Normofertile treatment	4	5,5	2,5	4,5	3	3	3,5	2,5	3,5	3	3,5 ± 0,942

Values are expressed as individual ages for each subject, followed by the mean and standard deviation (SD) for each group.
SC: subfertile control; NC: normofertile control; ST: subfertile treatment; NT: normofertile treatment.

of fatty acids (Lenzi et al. 2000). This high content of unsaturated fatty acids makes sperm particularly sensitive to oxidative effects (Zalata et al. 1998; Aitken et al. 2014). ROS plays a crucial role in the signalling regulation of the transduction cascade that controls sperm maturation and functional capacity (Vernet et al. 2004). However, at high concentrations, ROS can be devastating to cell function by causing excessive peroxidation of membrane phospholipids, resulting in damaging effects on both nuclear and mitochondrial DNA (Aitken and Baker 2006).

Therefore, a promising option to improve long-term semen preservation is the use of antioxidants that regulate ROS levels ensuring only the minimum amount necessary to maintain normal sperm function (Maneesh and Jayalekshmi 2006; Ciani et al. 2021; Silvestre et al. 2021). The use of antioxidant supplements has been suggested to mitigate the effects of oxidative stress during the storage process of canine sperm, helping to slow or prevent its deterioration (Michael et al. 2007; Beccaglia et al. 2009; Michael et al. 2009; Kmenta et al. 2011; Del Prete et al. 2018a).

Lepidium meyenii, also known as Maca, is a plant of the Brassicaceae family that is common in Peru, North America and Europe (Gonzales et al. 2001). It has become very popular due to its pharmacological properties, including antimicrobial, antioxidant and anti-inflammatory activities (Półtorak et al. 2018; Mohammadbeigi et al. 2019). Maca is rich in valuable nutrients and various secondary metabolites such as macamides, alkaloids and glucosinolates (Canales et al. 2000), which reduce free radicals and protect cells from oxidative stress. Maca's effects on semen quality, spermatogenesis, sperm count and sperm motility in various species have been reported in numerous studies (Gonzales et al. 2001; Del Prete et al. 2018b; Aoki et al. 2019). These effects have been observed both in healthy animals (Canales et al. 2000; Gonzales et al. 2001; Inoue et al. 2016; Del Prete et al. 2018b; Gattuso et al. 2024) and in animals with induced subfertility (Valdivia et al. 2016; Onaolapo et al. 2018).

The aim of this study was to assess the potential beneficial effect of oral supplementation with Black Maca (*Lepidium meyenii*) on improving the quality of fresh sperm and, for the first time, on maintaining it during cooling storage, as well as to investigate its effect on changes in serum testosterone concentrations.

Materials and methods

Ethical approval

All treatments, housing and animal care followed EU Directive 2010/63/EU on the protection of animals used for scientific purposes. The Ethics Committee of the Department of Veterinary Medicine and Animal Productions at the University of Messina, Italy (prot. no. 09/2023 ter), approved the protocol and procedures. Informed consent was obtained from each dog owner before its inclusion in the study.

Animals and study design

A total of 40 client-owned healthy male dogs, heterogeneous in breed and age, were included in the study. The subjects were aged between 2 and 6 years, with a mean age of 3.54 ± 1.2 years (Table 1), and their weight ranged from 19 kg to 35 kg, with a mean of 25.80 ± 9.2 kg. Inclusion criteria for the animals were based on clinical history, physical examination, reproductive ultrasound of the prostate and testes, and semen parameters such as ejaculate volume, total sperm concentration, total and progressive motility and morphology. To minimise defects in semen stored in the epididymis, such as reduced motility and increased debris, a preliminary semen collection was performed the day before the examination. Subjects had to be without clinically relevant systemic disorders, fed a specific commercial diet free of additives such as L-arginine and/or vitamins and antioxidants that could interfere with the study. The maintenance diet for adult dogs had chicken as the first ingredient and its formulation was characterised by digestibility and palatability due to the inclusion of fresh meat. This diet contained 26% crude protein, 2.2% crude fibre, 14% crude fat, 6.5% crude ash, 1.2% calcium, 1% phosphorus, 0.7% n-3 fatty acids, and 5.2% n-6 fatty acids. Finally, the animals had to be housed in rooms with adequate natural light.

Subjects who had semen parameters incompatible with adequate reproductive capacity (total and progressive motility and morphology values are less than 60%) and who had experienced at least one reproductive failure in the six months prior to the study, either by natural mating or artificial insemination, were classified as subfertile ($n = 20$).

Subjects with semen parameters compatible with adequate reproductive capacity (total and progressive motility and morphology values are above 60%) and a normal reproductive history in the six months prior to the study, with numerically representative litter size by both natural mating and artificial insemination, were classified as normofertile ($n = 20$).

Subjects in both subfertile ($n = 20$) and normofertile ($n = 20$) groups were further randomly divided into a control group ($n = 10$) and a treatment group ($n = 10$). The subjects were then divided into a subfertile control group (SC group) ($n = 10$), a subfertile treatment group (ST group) ($n = 10$), a normofertile control group (NC group) ($n = 10$), and a normofertile treatment group (NT group) ($n = 10$).

Subjects in the treatment groups (ST and NT groups) received an oral black Maca supplement in capsule form at a dose of 75 mg/kg [Maca extract 10:1, thickening agent (hydroxypropyl methylcellulose), and ground rice]. The supplement was given once daily during the main meal throughout the study (62 days). The galenic preparation of the maca extract supplement was carried out using crude black maca extract powder (Erbavoglio™). Subjects in the control groups (SC and NC groups) received a placebo consisting of a starch-only capsule.

Three semen samples were collected from each subject at three time points of the sperm cycle, which lasts approximately 62 days in dogs (Soares et al. 2009), for a total of 120 samples throughout the study. Samples were collected immediately before the start of oral supplementation (T_0), after 31 days (T_{31}), and after 62 days (T_{62}). Blood samples were taken from the cephalic vein of each dog included in the study each day (T_0, T_{31}, and T_{62}) between 8:00 and 10:00 am prior to semen collection to assess serum testosterone levels. Blood samples were collected in sterile glass tubes for hormonal analyses. For these purposes, the serum was centrifuged within 60 min of collection and refrigerated at 4 °C for hormonal analyses, which were performed within 24 h of collection at the Veterinary Diagnostic Centre BIOGENE (Catania, Italy). Serum testosterone concentrations were assayed using an immunoassay system based on the Enzyme Linked Fluorescent Assay (ELFA) principles by Mini VIDAS system (BioMerieux S.A., Lyon, France).

Sampling procedures and semen analysis

Sperm collection was performed in a quiet and appropriate environment with a non-slip floor, by manual collection and in the presence of a teasing bitch. Semen evaluation was carried out according to the indications in the literature and included the assessments commonly used in clinical practice for semen quality analysis (Tanga et al. 2021).

The ejaculate was fractionated by discarding the third fraction and immediately examining the first two fractions. Sperm concentration was assessed freshly with an SDM1 photometer (MiniTube™), by placing 10 µl of ejaculate in the appropriate loggia of the instrument's analysis microscope.

Motility was assessed by CASA software. The analysis was performed with the following parameters: frame acquired 30, frame rate 60 Hz, minimum cell contrast 75, minimum cell size 4 pixels, straightness threshold 75%, path velocity threshold 100 µm/$s-1$ average path velocity (VAP) cut-off 9.0 µm/s^{-1}, medium VAP cut-off 20 µm/s^{-1}, head size non-motile 4 pixels, head intensity non-motile 80, static head size 0.44–4.98, static head intensity 0.49–1.68 and static elongation 17%–96%.

After staining with eosin/nigrosine, cell morphology was assessed by examining at least 200 spermatozoa per slide.

To analyse the integrity of the sperm membrane, the hypo-osmotic swelling (HOS) test was performed. A drop of semen (50 µL) from each subject was mixed with 0.5 mL of HOS solution and incubated at 37 °C for 30 min. A 5 µL drop of the mixture was placed on a pre-warmed clean slide and 200 spermatozoa were examined for swelling within 5–10 min by phase-contrast microscopy (Eclipse Ts2, Nikon, Minato-Ku, Tokyo, Japan). Swelling was indicated by coiling of the spermatozoa tail and these spermatozoa were considered to have an intact plasma membrane.

An aliquot of the previously analysed fresh semen was centrifuged at 1800 rpm (700× g) for 5 min and the supernatant seminal plasma was removed to optimise storage at +5 °C. The remaining pellet was diluted appropriately with CaniPlus Chill ST extender (Minitüb, Germany). Sperm dilution with the extender was modulated according to the initial sperm concentration of each subject. Samples with a high sperm concentration were diluted 1:3, whereas samples with a lower concentration were diluted 1:1. The samples were immediately refrigerated and stored at +5 °C for later evaluation. Total motility, progressive motility and membrane integrity were assessed at 3 (T_{3h}), 24 (T_{24h}), 48 (T_{48h}), and 72 h (T_{72h}). The decrease in total and progressive sperm motility and membrane integrity was calculated by subtracting the values recorded at T_{72h} from those measured at T_{3h}. The result was then expressed as a percentage decrease from the baseline value, allowing quantification of the decrease in motility and membrane integrity through time.

Statistical analysis

Data were analysed using SAS software (version 9.4; SAS Institute). Normality of data was checked by using the univariate procedure of SAS. All data were subjected to ANOVA using mixed models for repeated measures (GLIMMIX Procedure; SAS Institute). The statistical model included treatment (Treatment and Control), fertility (Normofertile, Subfertile), day (T_0, T_{31}, and T_{62}) and hour (T_{3h}, T_{24h}, T_{48h}, and T_{72h}), their interaction as fixed effects, whereas dogs were included as random effect. The Kenward-Roger

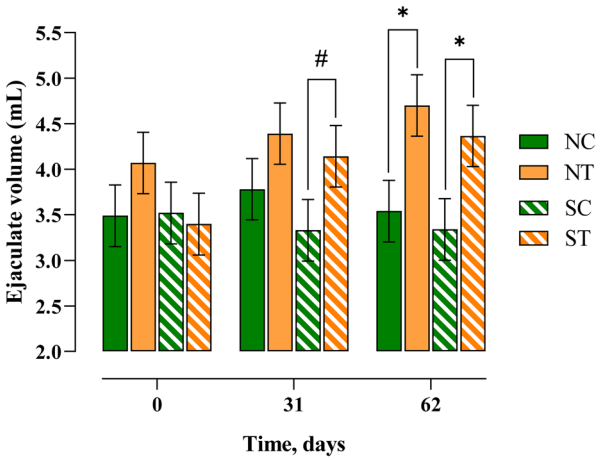

Figure 1. Ejaculate volume (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

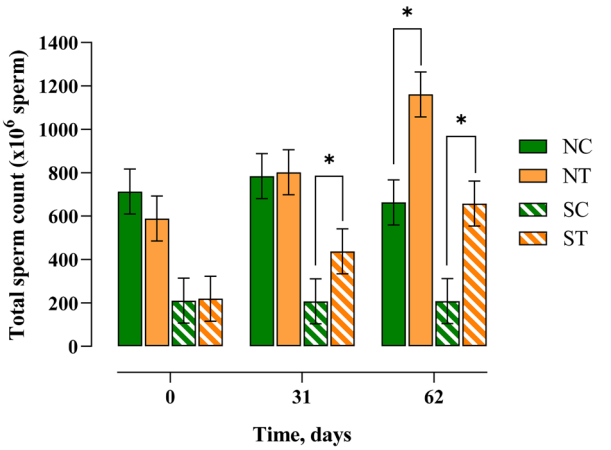

Figure 2. Total sperm count (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

statement was used to compute the denominator degrees of freedom, whereas spatial power was used as the covariance structure. Pairwise comparisons were performed by Tukey's post hoc tests. Comparisons with $p \leq 0.05$ were considered significant, whereas when $0.10 \geq p \geq 0.05$ they were discussed in the context of tendencies.

Results

Fresh semen-quantitative parameters

In subfertile subjects, ejaculate volume at T_0 was similar in the treatment (ST) and control (SC) groups, with mean values of 3.39 ± 1.36 mL and 3.52 ± 0.73 mL, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (4.14 ± 1.30 mL *vs* 3.33 ± 0.69 mL) at T_{62} (4.36 ± 1.57 *vs* 3.34 ± 0.58 mL; $p < 0.05$) compared to the subjects in the control group (SC) (Figure 1).

In normofertile subjects, ejaculate volume at T_0 was also comparable in the treatment (NT) and control (NC) groups, with mean values of 4.06 ± 0.66 mL and 3.49 ± 1.32 mL, respectively. After oral Maca supplementation, subjects in the treatment group (NT) showed higher values at both T_{31} (4.39 ± 0.62 mL *vs* 3.78 ± 1.34 mL) at T_{62} (4.7 ± 0.59 *vs* 3.54 ± 1.21 mL; $p < 0.05$) compared to the subjects in the control group (SC) (Figure 1).

In subfertile subjects, the total sperm count at T_0 was similar in the treatment (ST) and control (SC) groups, with mean values of $219.77 \pm 135.56 \times 10^6$ spz and $209.93 \pm 62.52 \times 10^6$ spz, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} ($437.61 \pm 290.83 \times 10^6$ spz *vs* $206.91 \pm 58.21 \times 10^6$ spz; $p < 0.05$) at T_{62} ($657.68 \pm 359.88 \times 10^6$ spz *vs* $208.29 \pm 59.27 \times 10^6$ spz; $p < 0.05$) compared to the subjects in the control group (SC) (Figure 2).

In normoferile subjects, the total sperm count at T_0 was similar in the treatment (NT) and control (NC) groups, with mean values of $588.78 \pm 239.15 \times 10^6$ spz and $712.73 \pm 526.73 \times 10^6$ spz, respectively. After oral Maca supplementation, in normofertile subjects in the treatment group (NT), the total sperm count showed higher values at both T_{31} ($802.01 \pm 263.74 \times 10^6$ spz *vs* $783.98 \pm 597.99 \times 10^6$ spz) at T_{62} ($1160.77 \pm 363.37 \times 10^6$ spz *vs* $663.31 \pm 389.53 \times 10^6$ spz; $p < 0.05$) compared to the subjects in the control group (NC) (Figure 2).

Fresh semen-motility, morphology, and membrane integrity

The total motility at T_0 of the subfertile subjects in the treatment (ST) and control (SC) groups was similar, 56.2 ± 12.3% and 56.7 ± 10.80%, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (73.2 ± 7.39% *vs* 57.5 ± 11.64%; $p < 0.001$) at T_{62} (82.8 ± 8.67%*vs* 57 ± 12.04%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 3).

In subjects with normal fertility in the treatment (NT) and control (NC) groups, total motility was also similar at T_0, with values of 80.5 ± 3.2% and 83.1 ± 5.23%, respectively. After oral Maca supplementation, subjects in the treatment group (NT) showed higher values at both T_{31} (84.4 ± 2.11% *vs* 82.1 ± 6.10%) at T_{62} (88.7 ± 3.52% *vs* 83.3 ± 5.59%) compared to the subjects in the control group (NC) (Figure 3).

The progressive motility at T_0 of subfertile subjects in the treatment (ST) and control (SC) groups was similar, with values of 53.5 ± 12.58% and 51.6 ± 9.84%, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (67.7 ± 8.75% *vs* 51.36 ± 9.09%; $p < 0.001$) at T_{62} (77 ± 9.77% *vs* 51.32 ± 9.52%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 4).

In normofertile subjects in the treatment (NT) and control (NC) groups, progressive motility was similar at T_0, with values of 78.6 ± 2.22% and 71.7 ± 16.99%, respectively. After oral Maca supplementation,

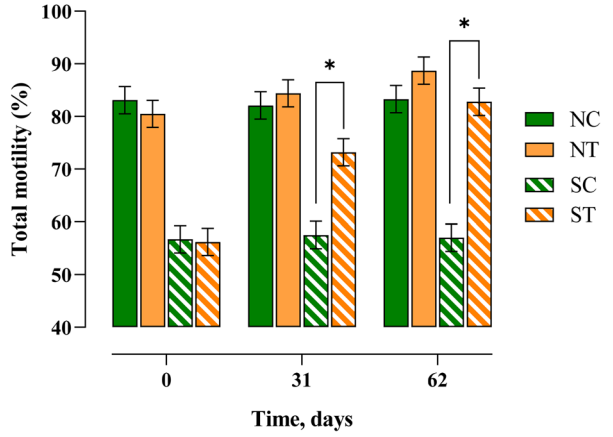

Figure 3. Total motility (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

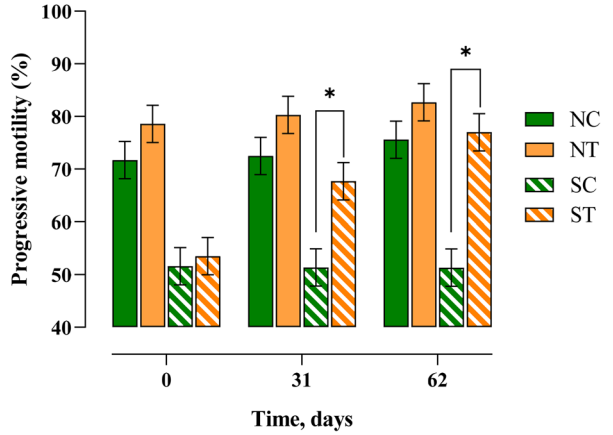

Figure 4. Progressive motility (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

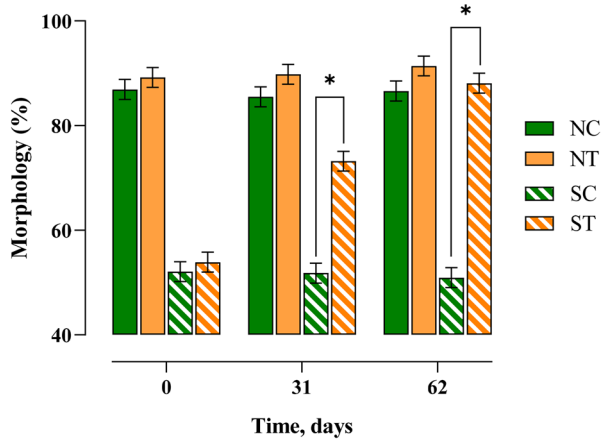

Figure 5. Morphology (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

subjects in the treatment group (NT) showed higher values at both T_{31} (80.3 ± 2.26% *vs* 72.5 ± 18.45%) at T_{62} (82.7 ± 2.90% *vs* 75.6 ± 16.01%) compared to the subjects in the control group (NC) (Figure 4).

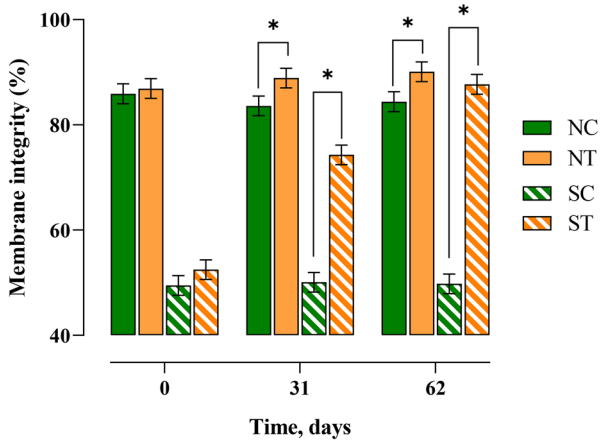

Figure 6. Membrane integrity (mean ± SD) of semen collected at T_0, T_{31}, and T_{62} in subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

The percentage of morphologically normal spermatozoa at T_0 of the subfertile subjects in the treatment (ST) and control (SC) groups was also comparable, with values of 53.9 ± 9.87% and 52.07 ± 6.70%, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (73.2 ± 8.37% *vs* 51.80 ± 5.32%; $p < 0.001$) at T_{62} (88.1 ± 3.21% *vs* 50.93 ± 7.29%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 5).

In normofertile subjects in the treatment (NT) and control (NC) groups, the percentage of morphologically normal spermatozoa was similar at T_0, with values of 89.2 ± 5.47% and 86.9 ± 4.09%, respectively. After oral Maca supplementation, subjects in the treatment group (NT) showed higher values at both T_{31} (89.8 ± 4.39% *vs* 85.5 ± 5.08%) at T_{62} (91.4 ± 3.89% *vs* 86.6 ± 4.67%) compared to the subjects in the control group (NC) (Figure 5).

The membrane integrity at T_0 of the subfertile subjects in the treatment (ST) and control (SC) groups was similar, 52.5 ± 9.51% and 49.5 ± 6.46%, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (74.3 ± 8.24% *vs* 50.1 ± 5.40%; $p < 0.001$) at T_{62} (87.7 ± 3.40% *vs* 49.8 ± 6.89%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 6).

In subjects with normal fertility in the treatment (NT) and control (NC) groups, membrane integrity was also similar at T_0, with values of 86.9 ± 5.44% and 85.9 ± 4.55%, respectively. After oral Maca supplementation, subjects in the treatment group (NT) showed higher values at both T_{31} (88.9 ± 5.13% *vs* 83.6 ± 4.69%; $p < 0.05$) at T_{62} (90.1 ± 4.01% *vs* 84.4 ± 4.55%; $p < 0.05$) compared to the subjects in the control group (NC) (Figure 6).

Cooled semen

Total and progressive motility decreased significantly during cold storage in all groups.

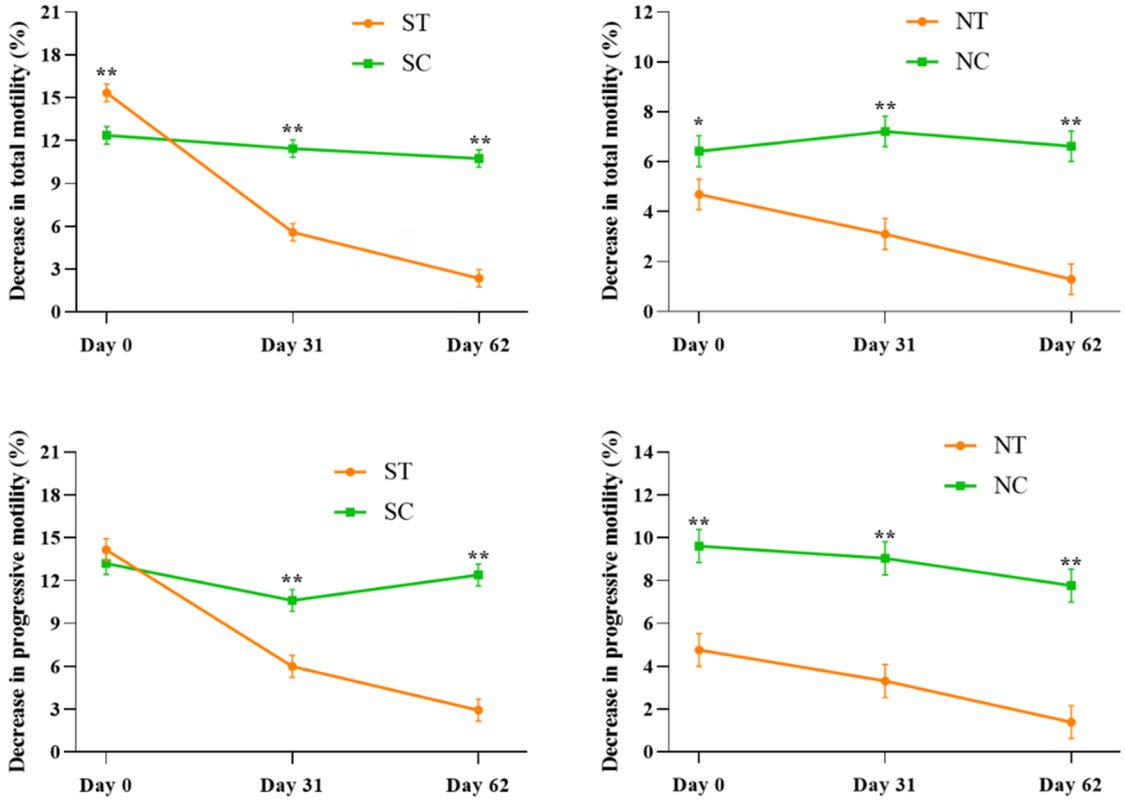

Figure 7. Daily percentage difference in total and progressive semen motility for treatment (ST and NT) and control (SC and NC) groups during storage at 5 °C for each day of collection (*$p < 0.05$; **$p < 0.001$).

At T_0, the decrease in total motility was significantly different between the ST and SC groups (15.33% *vs* 12.36%; $p < 0.001$) and between the NT and NC groups (4.69% *vs* 6.42%; $p < 0.05$).

After oral Maca supplementation, subfertile subjects in the treatment group (ST) showed lower values of decrease at both T_{31} (5.56% *vs* 11.43%; $p < 0.001$) at T_{62} (2.35% *vs* 10.72%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 7).

After oral Maca supplementation, normofertile subjects in the treatment group (NT) showed lower values of decrease at both T_{31} (3.10% *vs* 7.21%; $p < 0.001$) at T_{62} (1.29% *vs* 6.62%; $p < 0.001$) compared to the subjects in the control group (NC) (Figure 7).

At T_0, the decrease in progressive motility was significantly different between NT and NC groups (4.75% vs 9.61%; $p < 0.001$). However, the decrease in progressive motility was similar between the ST and SC groups (14.17% vs 13.20%).

After oral Maca supplementation, subfertile subjects in the treatment group (ST) showed lower values of decrease at both T_{31} (5.98% *vs* 10.61%; $p < 0.001$) at T_{62} (2.93% *vs* 12.39%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 7).

After oral Maca supplementation, normofertile subjects in the treatment group (NT) showed lower values of decrease at both T_{31} (3.31% *vs* 9.04%; $p < 0.001$) at T_{62} (1.37% *vs* 7.76%; $p < 0.001$) compared to the subjects in the control group (NC) (Figure 7).

In all four groups, storage of the semen of the included subjects at refrigeration temperatures resulted in a significant decrease in membrane integrity. At T_0, the decrease in in membrane integrity was significantly different between the ST and SC groups (10.53% vs 13.69%; $p < 0.001$) and between the NT and NC groups (4.83% vs 6.81%; $p < 0.05$).

After oral Maca supplementation, subfertile subjects in the treatment group (ST) showed lower values of decrease at both T_{31} (5.64% *vs* 12.76%; $p < 0.001$) at T_{62} (2.73% *vs* 14.15%; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 8).

After oral Maca supplementation, normofertile subjects in the treatment group (NT) showed lower values of decrease at both T_{31} (3.15% *vs* 6.21%; $p < 0.001$) at T_{62} (1.27% *vs* 5.89%; $p < 0.001$) compared to the subjects in the control group (NC) (Figure 8).

Testosterone

The testosterone value at T_0 of the subfertile subjects in the treatment (ST) and control (SC) groups was similar, 1.66 ± 0.38 ng/mL and 1.74 ± 0.30 ng/mL, respectively. After oral Maca supplementation, subjects in the treatment group (ST) showed higher values at both T_{31} (2.84 ± 0.42 ng/mL *vs* 1.63 ± 0.35 ng/mL; $p < 0.001$) at T_{62} (4.05 ± 0.40 ng/mL *vs* 1.61 ± 0.39 ng/mL; $p < 0.001$) compared to the subjects in the control group (SC) (Figure 9).

At T_0, the testosterone value was significantly different between the NC and NT groups, with values of 4.68 ± 0.48 ng/mL and 4.04 ± 0.40 ng/mL, respectively ($p < 0.001$). After oral Maca supplementation, subjects in the treatment group (NT) showed higher

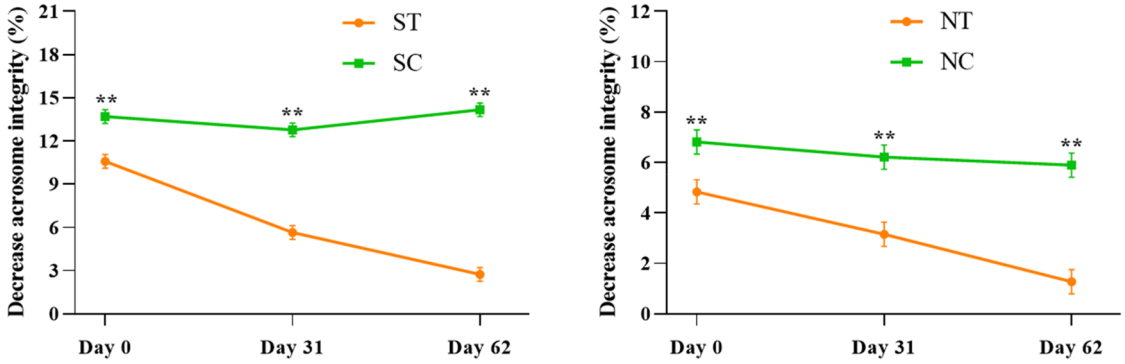

Figure 8. Daily percentage difference in semen membrane integrity for treatment (ST and NT) and control (SC and NC) groups during storage at 5 °C for each day of collection. (*$p < 0.05$; **$p < 0.001$).

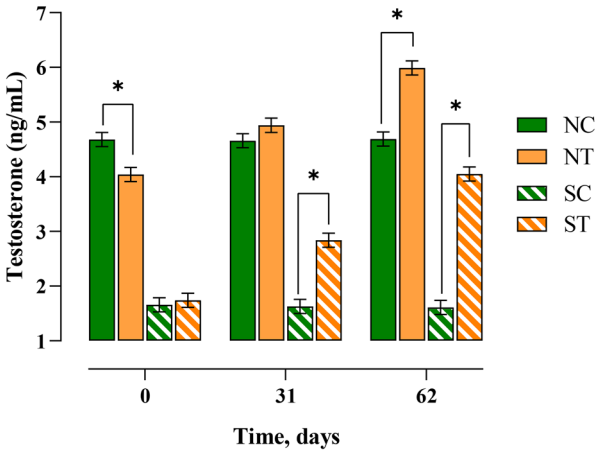

Figure 9. Serum testosterone concentrations (mean ± SD) of blood collected at T_0, T_{31}, and T_{62} in the subfertile treatment group ($n = 10$), subfertile control group ($n = 10$), normofertile treatment group ($n = 10$), and normofertile control group ($n = 10$; *$p < 0.05$).

values at both T_{31} (4.94 ± 0.46 ng/mL *vs* 4.66 ± 0.45 ng/mL) at T_{62} (5.99 ± 0.37 ng/mL *vs* 4.69 ± 0.45 ng/mL; $p < 0.001$) compared to the subjects in the control group (NC) (Figure 9).

Discussion

Free radicals, which are naturally produced in the organism, provide vital functions in immune defence and cell signalling (Martemucci et al. 2022). However, when free radical levels exceed the organism's antioxidant capacity, a state of oxidative stress can be induced, causing cellular damage and alterations in biochemical functions (Phaniendra et al. 2015).

In an attempt to prevent the harmful effects of oxidative stress, numerous studies have investigated the role of plants and fruits with antioxidant properties, including *Lepidium meyenii* (Maca), a plant of the Brassicaceae family native to the Peruvian Andes. Maca has been used for centuries by the Inca culture as an energiser, aphrodisiac and treatment for various conditions such as respiratory disorders, anaemia and rheumatism, and today is the subject of growing interest for its potential protective effect against oxidative cell damage (Sifuentes-Penagos et al. 2015; Beharry and Heinrich 2018; Tang et al. 2019; Orhan et al. 2022). Maca root contains several bioactive compounds, including macamides, macaenes, polysaccharides, polyphenols, and glucosinolates, with recognised effects on oxidative balance (Gonzales et al. 2014; Lamou et al. 2016; Liu et al. 2023).

Shehab et al. (2024) provided further evidence of Maca's properties by comparing them with other plants known to support male reproductive health, such as *Trigonella foenum-graecum* and *Tribulus arabica*. Among these, *Lepidium meyenii* showed a 63% inhibition of free radicals, making it particularly effective in reducing oxidative stress and improving sperm quality.

Various dietary supplementation protocols have evaluated the daily supplementation of different micronutrients such as selenium, vitamin E, coenzyme Q10 (CoQ10), zinc, folic acid and omega-3 polyunsaturated fatty acids (PUFA) to improve sperm quality in dogs. Supplementation with selenium and vitamin E improved sperm quality in dogs with reduced fertility (Domosławska et al. 2018) and helped four infertile dogs to mate successfully (Domosławska et al. 2019). Another study found no improvement in normospermic Cairn Terriers after supplementation with both micronutrients, probably due to the higher doses, which may have caused subclinical toxicity instead of a beneficial effect (Kirchhoff et al. 2017). The importance of the amounts of micronutrients to be supplemented in the diet is therefore crucial. In our study, the dose used (75 mg/kg) was chosen on the basis of previous reports in the literature (Gattuso et al. 2024). Supplementation with CoQ10 in aging dogs with poor semen quality resulted in improved sperm motility, reduced numbers of morphologically abnormal sperm and increased SOD activity in seminal plasma (Kobayashi et al. 2021). Another important factor to consider in dietary supplementation is the combination of multiple micronutrients and the synergistic interaction of all the molecules involved. In a study by Alonge et al. a commercial dry diet containing a complex of vitamin E (250 mg/kg), selenium (0.27 mg/kg), zinc (180 mg/kg), folic acid (1.5 mg/kg), and omega-3 polyunsaturated fatty acids (5 g/kg) increased sperm count and improved ejaculate motility and membrane properties in healthy normospermic dogs (Alonge et al. 2019). In another study by Ciribé et al.

a nutraceutical diet with a balanced omega-6:omega-3 ratio (4:1), enriched with zinc (50 mg/kg), *Lepidium meyenii* (865 mg/kg), Tribulus terrestris (52 mg/kg), l-carnitine (420 mg/kg), beta-carotene (230 mg/kg), vitamin E (240 mg/kg), and folic acid (0. 27 mg/kg), increased motility percentage, semen volume and concentration, and total number of sperm per ejaculation in dogs suffering from infertility (Ciribé et al. 2018).

Recently, interest in the use of Maca as a dietary supplement has increased, with numerous studies investigating its positive effects on reproductive health in both men and women (Melnikovova et al. 2015; Stojanovska et al. 2015). Maca has also attracted attention in the veterinary field, with several studies analysing its effects on ruminants (Clément et al. 2010; 2012), showing improvements in semen quality and an increase in the number of copulations and ejaculations after supplementation with maca extract. Similar studies have been carried out in various species, including stallions (Del Prete et al. 2018b), dogs (Gattuso et al. 2024), rabbits (El-Sheikh et al. 2019), poultry (Korkmaz et al. 2016; Turgud and Narinç 2022), and mice (Gonzales et al. 2001b; Valdivia et al. 2016; Onaolapo et al. 2018). In studies on mice and rats, an improvement in male libido was observed (Zheng et al. 2000; Cicero et al. 2001), while in men Maca supplementation showed positive effects on sexual libido without altering hormone levels (Gonzales et al. 2002). In addition, a study in breeding bulls (Matos 1995) reported a significant increase in sperm motility and sperm count after Maca supplementation. Other studies have suggested that Maca may increase sperm production in men (Gonzales et al. 2001a), improve testicular and epididymal weight in rats (Gonzales et al. 2001b), and reduce spermatogenetic damage caused by adverse environmental conditions such as high altitude in rats (Gonzales et al. 2004). Finally, studies have shown that Maca supplementation can improve spermatogenesis following damage caused by malathion poisoning (Bustos-Obregon et al. 2005). This is the first study to evaluate the preservation of semen parameters (total and progressive motility and membrane integrity) under refrigerated conditions and testosterone levels in dogs treated with oral supplementation of black maca (*Lepidium meyenii*).

There are currently no studies in the literature that report the maximum tolerated dose, the recommended dose, or any adverse effects associated with excessive or chronic consumption of this substance in dogs. However, some preclinical studies in other species have evaluated the *in vivo* toxicity of oral administration, suggesting a low level of acute oral toxicity. In particular, pregelatinised maca has shown an LD50 greater than 7.5 g (Meissner et al. 2006a) and 15 g/kg/day in mice (Ulloa Del Carpio et al. 2024) and greater than 5 g/kg/day in rats (Meissner et al. 2006b). Therefore, short-term and long-term (90 days) oral administration of Maca as a dietary supplement or as a component of functional dietary and therapeutic preparations appeared to be safe.

For this reason, the authors of this study used a previously clinically tested dosage and duration (60 days) for which no adverse effects were reported.

The results for fresh semen are in agreement with those reported by Gattuso et al. (2024), confirming the improvement in sperm volume, concentration, motility and morphology after oral Maca supplementation in dogs. Furthermore, our study introduces the assessment of membrane integrity also in fresh semen, providing a more complete view of sperm quality and cell viability.

In our study there was a significant improvement in the seminal parameters of fresh semen in dogs treated with oral Maca supplementation. Ejaculate volume increased significantly at T_{62} in both treatment groups (ST and NT). A significant increase in sperm concentration was observed, with the ST group showing an increase of approximately three times and the NT group an increase of approximately two times compared to their respective control groups. In the ST group, this improvement became statistically significant as early as T_{31}, suggesting a faster and more pronounced effect in this category of subjects. These results are consistent with what has been reported in the literature in adult rats (Gonzales et al. 2004, 2006), peripuberal bulls (Clément et al. 2010), dogs (Gattuso et al. 2024), and humans (Gonzales et al. 2001), where Maca supplementation led to similar increases in sperm concentration in different species.

Another important finding was the improvement in both total and progressive motility after Maca supplementation in subfertile dogs. This enhancement enabled the subfertile subjects to reach the physiological ranges for the species. Sperm morphology improved slightly in the NT group and significantly in the ST group. In the latter group, the increase became statistically significant as early as T_{31}, suggesting a beneficial effect of Maca supplementation on the morphological quality of spermatozoa in subfertile subjects.

Finally, sperm membrane integrity increased in both treatment groups (NT and ST). However, in the subfertile group, the increase in membrane integrity was much more pronounced, indicating a more significant and consistent improvement in these subjects.

In our study, oral Maca supplementation led to a significant increase in serum testosterone levels in dogs, a result that is only partially consistent with the evidence reported in the literature. In fact, previous studies have shown that the administration of Maca, especially the yellow and black varieties, was associated with an increase in sperm count and motility (Gonzales et al. 2013b) and stimulation of spermatogenesis (Clément et al. 2010, 2012), but without modifying testosterone levels (Balick and Lee 2002; Melnikovova et al. 2015). However, there are several studies in the literature that have reported an increase in the serum concentration of this hormone. The addition of Maca powder to the diet increased serum testosterone concentrations in male quails

(Gül et al. 2022; Olgun et al. 2022), rats (Gonzales et al. 2005), and mice (Oshima et al. 2003; Onaolapo et al. 2018). In one study, the addition of different Maca powders (a combination of yellow, black and red Maca) for 6 weeks in young male Wistar rats increased serum testosterone levels (Ohta et al. 2016). These findings suggest that the synergistic effect of saponin, arginine, lead, vitamin E, and all the complex phytochemicals contained in Maca may directly stimulate Leydig cells, especially in the metabolic process of cholesterol (Gül et al. 2022). Indeed, the study by Ohta et al. (2016) showed an increase in the serum concentration of this hormone in rats treated with a Maca extract, with no change in LH levels. The authors suggested that this effect was mediated by an increased ability of Leydig cells to produce testosterone. This result could in fact be related to the antioxidant properties of Maca which, by reducing oxidative stress, preserve the functionality of the Leydig cells and improve their efficiency in synthesising testosterone. Our results suggest that the effect of Maca on increasing serum testosterone in dogs may be due, at least in part, to its antioxidant action, which directly supports and stimulates the activity of Leydig cells, promoting their function and thus contributing to increased testosterone levels.

The results for cooled semen highlight the positive effect of oral Maca supplementation in preserving semen quality during refrigerated storage. The decrease in total and progressive motility during storage is an expected observation due to the metabolic and structural changes induced in spermatozoa by low temperatures. However, in the Maca treatment groups (ST and NT), the percentage decrease in these parameters was significantly lower compared to the beginning of the supplementation period. This observation suggests that Maca has a protective effect, probably mediated by an antioxidant action that reduces oxidative damage typical of storage in refrigerated conditions. In contrast, in the control groups (SC and NC), the percentage decrease in total and progressive motility remained unchanged, indicating the absence of a similar protective effect.

Similarly, membrane integrity decreased significantly in all groups during refrigeration, a phenomenon associated with increased lipid peroxidation and loss of plasma membrane function. However, in the treated groups (ST and NT), the decrease in membrane integrity was significantly lower than at the beginning of supplementation. This result supports the hypothesis that the bioactive components of Maca may contribute to the maintenance of membrane integrity through mechanisms of lipid stabilization and reduction of oxidative stress. In the control groups (SC and NC), in contrast, no significant reduction in damage was observed during the supplementation period.

Our results are in agreement with those reported by Del Prete et al. (2018b), who first showed that dietary supplementation with Maca improves the quality of equine semen, increasing its resistance to refrigeration and storage. Specifically, the study in stallions showed an improvement in total and progressive motility, as well as preservation of acrosome and DNA integrity during refrigerated storage. Similarly, in our Maca-treated groups (ST and NT) we observed significantly less reduction in total and progressive motility and better membrane integrity than in the control groups, showing a protective effect of Maca on cooled semen.

These findings may be related to certain components of Maca, such as polysaccharides and polyphenols, which have protective properties against oxidative damage through their effects on cellular signalling pathways, including Nrf2 and MAPK, and by enhancing the antioxidant response through the activation of enzymes such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Korkmaz 2018; Lee and Park 2021; Fei et al. 2022; Mason et al. 2022). Some maca alkaloids also modulate antioxidant enzyme activity, thereby protecting reproductive tissues from lipid peroxidation that compromises the integrity of cell membranes rich in polyunsaturated fatty acids (PUFA) (Korkmaz 2018). Since sperm membranes require high levels of PUFA to provide the plasma membrane fluidity essential for fertilisation processes (Wathes et al. 2007), oxidative stress-induced changes in PUFA composition cause alterations in membrane architecture that alter membrane permeability and fluidity (Cross 2003; Maldjian et al. 2005). Therefore, although the exact mechanisms of action of Maca in improving semen quality are still unknown, the increase in the intrinsic resistance of ejaculated spermatozoa to lipid peroxidation could explain the overall improved sperm quality in subjects treated with Maca (Del Prete et al. 2018a; D'Anza et al. 2021; Shehab et al. 2024). The protective role of maca is further enhanced by the presence of components such as beta-sitosterol, which contributes to the regulation of the enzymatic antioxidant response and supports mitochondrial efficiency, demonstrating a synergistic effect that amplifies its protective action on tissues (Orhan et al. 2022).

Our results therefore showed, in both fresh and chilled semen, a clear improvement in sperm quality at T_{31} and T_{62}, in line with the duration of the seminiferous epithelial cycle, which is completed in approximately 62 days (Soares et al. 2009). This suggests that Maca supplementation may progressively improve spermatogenesis and sperm quality, with the effects becoming more pronounced over time. The most significant effect observed in subfertile subjects is attributed to the known antioxidant activity of Maca, which can counteract oxidative stress, a major cause of deterioration in sperm quality. Consequently, in normofertile subjects who already had optimal semen parameters, the margin for improvement was physiologically more limited.

This study has some limitations, including sample size and limited observation period. Although the data collected yielded significant results, these findings may not fully reflect long-term effects or individual variations. In addition, the molecular

mechanisms responsible for the observed effects, particularly those related to cellular oxidative stress pathways, were not investigated.

Future research should involve larger samples and longer observation periods to more accurately assess the duration and progression of benefits associated with Maca. To better understand the therapeutic potential of Maca in dogs, it will also be important to further investigate the mechanisms of action through molecular marker analysis and to compare the efficacy of Maca with that of other nutraceuticals.

Conclusion

In conclusion, this study demonstrates the efficacy of Black Maca (*Lepidium meyenii*) as an oral supplement to improve canine semen quality, with significant effects on ejaculate volume, concentration, motility, morphology, and sperm membrane integrity. In addition, its ability to preserve semen quality during storage at 5 °C highlights an important protective action of Maca against oxidative stress. The manifestation of these effects proved to be more pronounced in subfertile subjects compared to those classified as having normal fertility levels.

The observed increase in serum testosterone levels in dogs treated with Maca provides evidence that the treatment may have a direct effect on canine reproductive function. This hormonal increase, which is known to play a central role in spermatogenesis, may account for the improvement in seminal parameters observed in treatment subjects, particularly regarding increased sperm concentration. These results underline the clinical importance of Maca as a nutraceutical for treating subfertility and improving semen storage, making it a promising option for managing and improving reproductive performance in dogs.

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Authors' contributions

Conceptualization, V.Z., D.T.G, and M.Q.; methodology, D.T.G., V.Z., A.T., and T.C.; formal analysis, C.C., C.T., and M.T.; investigation, G.P., G.D., and A.T.; data curation, C.C., M.T., and C.T.; writing—original draft preparation, V.Z and M.Q.; writing—review and editing, G.P., G.D., A.T., and T.C.; supervision, M.Q. and A.T. All authors have read and agreed to the published version of the manuscript.

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The authors report there are no competing interests to declare.

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