Received: 16 October 2021 | Accepted: 2 March 2022

DOI: 10.1111/jpn.13705

ORIGINAL ARTICLE

Poultry

Journal of Animal Physiology and Animal Nutrition WILEY

Effect of dietary n-3 fatty acid and rooibos (*Aspalathus linearis*) supplementation on semen quality, sperm fatty acids and reproductive performance of aged male broiler breeders

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Abstract

The purpose of this study was to assess the effects of dietary fish oil (FO) and rooibos supplementation on semen quality, fatty acids composition and reproductive performance of aged male broiler breeders. Seventy-two 47-week-old Ross broiler breeder roosters were randomly assigned to a 2 × 3 factorial arrangements to include two FO concentrations (0% and 2%) and 3 rooibos concentrations (0%, 1.5% and 3%) for 13 weeks consecutive. The different diets affected semen parameters significantly ($p < 0.05$), except for the semen concentration and abnormality of the sperm. The sperm of the FO and 3% rooibos-treated group showed better motility and viability when compared to the other groups ($p < 0.05$). The susceptibility of semen to lipid peroxidation was increased in roosters fed the rooibos-free diets ($p < 0.05$), but it was reduced ($p < 0.05$) when the diet was supplemented with 1.5% and 3% rooibos. In addition, at 64 weeks, the highest concentration of testosterone was observed in the roosters fed a diet that included 2% FO and 3% rooibos ($p < 0.05$); however, the difference in testosterone levels between Week 52 and Week 64 was not significant ($p > 0.05$). The fertility rate of collected eggs from the FO and 3% rooibos group was higher ($p < 0.05$) than that of the other groups at the end of the experiment. In conclusion, dietary inclusion of FO along with rooibos improved seminal quality and reproduction performance in aged roosters.

KEYWORDS

aging, fertility, natural antioxidant, polyunsaturated fatty acids, rooster

1 | INTRODUCTION

Considering the low ratio of roosters to hens in the breeding flocks and the decline in the fertility of roosters after the age of 45 weeks, it is necessary to be attentive to the reproductive performance of roosters (Feng et al., 2015; Mangiagalli et al., 2010). The declined semen quality of the aged rooster is related to many factors including weight gain, reduced sperm and testosterone production, and a decline in semen antioxidant capacity (Khan, 2011). The lipid content of the plasma membranes of spermatozoa bears a functional role in the sperm's fertilizing capability (Cerolini et al., 1997) due to the high polyunsaturated fatty acids (PUFAs) content of sperm, but this also renders the spermatozoa extremely susceptible to lipid peroxidation in reaction to reactive oxygen species (ROS; Raei et al., 2021), which

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is closely associated with male infertility (Surai et al., 2001). Avian sperms due to having a higher content of PUFA than mammals are more susceptible to lipid peroxidation and therefore be more prone to reduced fertility (Raei et al., 2021). Lipid peroxidation results when intracellular ROS production overcomes the endogenous antioxidant defence mechanism used by cells, including sperm, and an immediate accumulation of lipid peroxides occurs in the plasma membrane (Neuman et al., 2002). The positive effects of docosahexaenoic acid (DHA) and eicosapentaenoic acid (EPA) have been reported to improve the plasma membrane of sperm and semen quality in different species (Asl et al., 2018a; Masoudi et al., 2016). As α-linolenic acid is a precursor of DHA and EPA in the body and fish oil (FO) is one of the common sources of this substance, the dietary inclusion of FO may improve semen quality by increasing the efficiency of DHA and EPA synthesis (Asl et al., 2018a; Brinsko et al., 2005). Also, many studies have shown that dietary supplementation of PUFAs, especially omega-3 (n-3) PUFAs, significantly increases sperm fertility in birds (Kelso et al., 1997; Zanussi et al., 2019). It seems that these contradictory results are mostly attributed to factors such as age, breed and dietary components such as the ratio of n-3:n-6 fatty acids in the diet (Asl et al., 2018b).

Accordingly, several approaches have been introduced in efforts to improve semen quality and the antioxidant capacity of seminal plasma in the roosters, including the use of dietary dried apple pomace (Akhlaghi, Ahangari, Zhandi, et al., 2014), lycopene (Mangiagalli et al., 2010), carnitine (Golzar Adabi et al., 2007), sage extract (Ommati et al., 2013), dried tomato pomace (Saemi et al., 2012), dried ginger rhizome (Akhlaghi, Ahangari, Navidshad, et al., 2014), *Satureja khuzistanica* powder (Heydari et al., 2015), vitamin E (Golzar Adabi et al., 2011), and camphor (Raei et al., 2021). Nevertheless, improving the seminal quality still appears to be a major concern in breeder production and the introduction of alternative approaches may be beneficial (Ommati et al., 2013).

Epidemiological studies have demonstrated an inverse correlation between the consumption of diets rich in flavonoids and decreased cellular damage via direct radical scavenging of ROS, chelating of metal ions or challenging enzymatic systems responsible for free radical production (Canda et al., 2014).

Aspalathus linearis (Brum.f) Dahlg. (Fabaceae), commonly known as rooibos, naturally grows in the Cederberg region in the Western Cape Province of South Africa and is commercially used as an herbal tea or tisane (Canda et al., 2014). During the summer months of January to April in the Southern hemisphere, the plant material is harvested and processed, by cutting the green harvested shoots into smaller pieces (~4 mm), bruising and wetting them, and allowing heap 'fermentation'/chemical oxidizing, sun drying and steam pasteurization to take place before packaging (https://sarooibos.co.za/faq/#toggle-id-6, accessed 3 February 2022 at 5:14 PM). The fermented rooibos does not contain caffeine and is considered low in tannin content when compared to the *Camellia sinensis* teas, rendering this herbal tea a very popular health beverage (Joubert & De Beer, 2011).

Polyphenolic constituents identified in rooibos include aspalathin (major polyphenol and unique only to rooibos), nothofagin, quercetin, rutin, isoquercitrin, orientin, luteolin, vitexin and chrysoeriol (Ajuwon et al., 2013). Rooibos herbal tea has been shown to possess potent antimutagenic (Marnewick et al., 2004), cancer-modulating (Marnewick et al., 2009), anti-inflammatory (Baba et al., 2009) and redox-modulating properties (Awoniyi et al., 2012). In addition, rooibos bioactivities include hepatoprotection, phytoestrogenic, antispasmodic, antihaemolytic, anti-aging, antimicrobial, antiviral, vasodilatory and improvement of the lipid profile, all of which may contribute to health improvement (Marnewick et al., 2004, 2005, 2011).

Although the antioxidant properties of rooibos have been well documented in human and animals, its effect on aged male broiler breeder fertility is yet to be investigated (Marnewick et al., 2004, 2005, 2011; Opuwari et al., 2020). Unprotected PUFA-enriched diets may cause the loss of sperm performance (Asl et al., 2018a). Therefore, the aim of this study was to evaluate the possible protective effects of fermented rooibos on semen quality and quantity, sperm fatty acids profile, testes weight and semen malondialdehyde (MDA) as an indication of the level of lipid oxidation damage in aged Ross 308 breeder roosters fed diets supplemented with FO as an n-3 fatty acid source (0% and 2%) and fermented rooibos (0%, 1.5% and 3%) as an exogenous antioxidant source.

2 | MATERIALS AND METHODS

2.1 | Birds and diets

In total, seventy-two 47-week-old Ross 308 broiler breeder roosters, of similar weight were randomly assigned to receive one of six dietary treatments (12 birds in each treatment group). Birds were housed individually in cages equipped with inside nipple drinkers and outside galvanized feeders and kept under uniform environmental conditions (15 L: 9D light timetable at 21–23°C ambient temperature).

Dietary treatments were randomly allocated to cages in a 2 × 3 factorial arrangement containing three fermented rooibos levels (R: 0, 15 and 30 g of rooibos per kg of diet) and 2 FO levels (F: 0% and 2% respectively). Birds were categorized into six groups and fed the following treatments: F0R0 = control diet with no rooibos and no FO; F0R1.5 = containing only 1.5% rooibos; F0R3 = containing only 3% rooibos; F2R0 = containing only 2% FO; F2R1.5 = containing 2% FO and 1.5% rooibos; and F2R3 = containing 2% FO and 3% rooibos. Roosters were fed daily by 132 g/day/bird at 47 weeks up to 145 g/day/bird at the beginning of the photoperiod (6:00 AM). The control and experimental diets in all trial groups were a standard commercial mash for Ross broiler breeder roosters (Table 1). The experimental diets in which bentonite was replaced by the rooibos were formulated to meet nutrient requirements of the broiler breeder roosters, as established by the Ross 308 parent stock nutrition specification (Aviagen, 2016).

The experimental rooibos contains 93.10% dry matter and supplied 4.30%, 1.05% and 1.55% of crude protein, ether extract and crude ash respectively. The fatty acids compositions of FO and experimental feeds are shown in Table 2. Quantification of the main

TABLE 1 Ingredients and chemical composition of experimental diets fed to breeder roosters (as-fed basis)

Item	Treatments					
	F0R0	F0R1.5	F0R3	F2R0	F2R1.5	F2R3
Ingredients						
Corn	671.67	672.47	673.26	577.61	578.41	579.21
Wheat bran	194.96	196.11	197.25	282.22	283.36	284.51
Soybean meal	74.11	72.08	70.06	61.37	59.34	57.31
Rooibos	0	15.00	30.00	0	15.00	30.00
Sand	30.00	15.00	0	30.00	15	0
Fish oil	0	0	0	20.00	20.00	20.00
Dicalcium phosphate	14.23	14.24	14.25	13.59	13.60	13.61
Limestone	7.07	7.07	7.07	7.24	7.24	7.24
Sodium bicarbonate	2.16	2.18	2.12	2.28	2.30	2.32
Salt	2.15	2.14	2.20	2.02	2.00	1.99
Mineral premix[a]	1.00	1.00	1.00	1.00	1.00	1.00
Vitamin premix[b]	1.00	1.00	1.00	1.00	1.00	1.00
Choline chloride 60%	0.60	0.63	0.68	0.60	0.65	0.70
HCl-Lysine	0.58	0.60	0.60	0.60	0.60	0.60
DL-Methionine	0.47	0.49	0.50	0.47	0.48	0.50
Total	1000	1000	1000	1000	1000	1000
Calculated chemical content						
Crude protein, %	11.31	11.55	11.62	11.41	11.39	11.33
Metabolisable energy, Kcal/kg	2700	2700	2700	2700	2700	2700
Crude fibre, 8%	4.076	4.085	4.094	4.816	4.825	4.834
Digestible Met, %	0.221	0.222	0.222	0.218	0.219	0.219
Digestible Lys, %	0.440	0.440	0.440	0.440	0.440	0.440
Digestible Met + Cys, %	0.420	0.420	0.420	0.420	0.420	0.420
Digestible Arg, %	0.622	0.618	0.613	0.635	0.631	0.626
Digestible Thr, %	0.340	0.338	0.336	0.336	0.334	0.332
Calcium, %	0.72	0.69	0.72	0.70	0.69	0.71
Available phosphorus, %	0.350	0.350	0.350	0.350	0.350	0.350
Sodium, %	0.180	0.180	0.180	0.180	0.180	0.180
Potassium, %	0.610	0.607	0.605	0.657	0.655	0.652
Chloride, %	0.230	0.230	0.230	0.230	0.230	0.230

Note: Values in parenthesis represent analysed contents of nutrients.

Abbreviations: F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos.

[a]Supplied per kilogram of diet: vitamin A, 14,000 IU; vitamin D3, 3000 IU; niacin, 50 mg; vitamin E, 30 mg; calcium pantothenate, 20 mg; vitamin K3, 4 mg; riboflavin, 7.0 mg; pyridoxine, 5.5 mg; folic acid, 1.5 mg; and biotin, 50 μg.

[b]Supplied per kilogram of diet: Fe ($FeSO_4 \cdot H_2O$), 85 mg; Mn ($MnSO_4 \cdot H_2O$), 90 mg; Zn (ZnO), 67.3 mg; Cu ($CuSO_4 \cdot 5H_2O$), 11 mg; and Se (Na_2SeO_3), 0.18 mg.

TABLE 2 Fatty acids composition of experimental diets and FO (% of total FA)

		Treatments						
		F0R0	F0R1.5	F0R3	F2R0	F2R1.5	F2R3	Fish oil
Myristic	14:0	1.38	1.32	1.44	2.04	2.11	2.27	3.39
Palmitic	16:0	27.26	27.65	26.37	23.02	23.16	24.05	22.1
Heptadecanoic	17:0	0.23	0.32	0.37	0.48	0.61	0.59	0.92
Stearic	18:0	3.51	3.24	3.54	3.14	3.09	3.33	4.61
Arachidic	20:0	0.56	0.57	0.41	0.35	0.41	0.38	0.21
Behenic	22:0	0.33	0.36	0.31	0.41	0.38	0.4	0.32
Lignoleric	24:0	1.07	1.20	1.16	0.04	0.02	0.05	0.5
Palmtoleic	16:1	1.15	0.96	1.19	2.65	2.45	1.89	4.59
Heptadecenoic	17:1	0.13	0.11	0.17	0.22	0.22	0.22	0.59
Oleic	18:1 n-9	30.69	30.85	31.12	31.95	31.44	30.73	33.14
cis-11-eicosenoic	20:1	0.24	0.28	0.22	0.4	0.38	0.29	1.62
Erucic	22:1 n-9	0.13	0.14	0.16	0.19	0.18	0.12	0.1
Nervonic	24:1	0.28	0.31	0.34	0.32	0.34	0.37	0.84
Linoleic	18:2 n-6	30.90	30.46	31.02	26.12	26.77	27.01	2.71
cis-11,14 eicosadienoic	20:2 n-6	0.003	0.008	0.006	0.007	0.004	0.005	0.004
cis-8,11,14 eicosatrienoic	20:3 n-6	0.04	0.09	0.03	0.08	0.07	0.04	0.15
Linolenic	18:3 n-3	1.41	1.38	1.42	1.57	1.61	1.61	1.03
EPA	20:5 n-3	0.46	0.52	0.48	1.78	1.82	1.59	5.8
DHA	22:6 n-3	0.05	0.02	0.04	5.09	4.73	4.86	17.26
Others		0.18	0.21	0.2	0.14	0.21	0.19	0.12
ΣSFA		34.34	34.66	33.6	29.48	29.78	31.07	32.05
ΣMUFA		32.62	32.65	33.2	35.73	35.01	33.62	40.88
ΣPUFA		32.86	32.48	32.99	34.65	35.00	35.115	26.95
n-6		30.94	30.56	31.05	26.21	26.84	27.055	2.86
n-3		1.92	1.92	1.94	8.44	8.16	8.06	24.09
n-6/n-3		16.12	15.92	16.01	3.11	3.29	3.36	0.12

Abbreviations: ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids; ΣSFA, total saturated fatty acids; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos; FA, fatty acid; FO, fish oil.

polyphenolic constituents of the fermented rooibos, the total polyphenol content and oxygen radical absorbance capacity are shown in Table 3.

2.2 | Semen assessments

During the adaptation period (48–50 weeks of age), the birds were trained by abdominal massage for semen collection (Burrows & Quinn, 1937). Semen was collected every 2 weeks between 52 and 64 weeks of age. The ejaculates from the birds ($n = 2$) were pooled and evaluated as a single sample. Semen volume was measured using graduated collecting tubes. Sperm concentration was determined after dilution of the semen sample (1:500 with saline), whereafter a droplet of semen was placed on the Neubaure hemocytometer (Golzar Adabi et al., 2011). The percentage of motile spermatozoa was determined by compound Olympus-BX50 light microscope (Olympus Optical) at ×100 magnification. For this, a 2–3 µl drop of diluted semen (1:100 in 2.9% sodium citrate solution) was placed on a warmed (40°C) microscopic slide and covered with a cover glass. At least six microscopic fields were examined. Each slide was evaluated twice (Golzar Adabi et al., 2011). Sperm viability and abnormality

TABLE 3 Quantification of the main polyphenolic constituents and antioxidant capacity of the fermented rooibos

HPLC-DAD μg/g rooibos							
Aspalathin	Orientin	Isoorientin	Isovitexin	Vitexin	Quercetin	Luteolin	Chrysoeriol
248.3 ± 8.5	332.1 ± 1.8	759.8 ± 9.6	114.0 ± 0.9	151.3 ± 2.1	45.3 ± 0.9	18.9 ± 0.1	10.6 ± 0.3
Total polyphenol content				28.2 ± 0.2 μmol GAE/g			
Oxygen radical absorbance capacity				482.6 ± 23.9 μmol TE/g			

Note: Values in columns: Average ± SD, with $n = 3$.

Abbreviations: GAE, gallic acid equivalent; HPLC-DAD, high-performance liquid chromatography with a diode-array detector; TE, trolox equivalents.

were evaluated, using 10 ml of diluted ejaculate (1:100 ml with saline) stained with 20 ml eosin–nigrosin solution and bright-field microscopy at ×100 magnification. Sperm displaying partial or complete purple staining were considered nonviable and only sperm showing strict exclusion of the stain were counted as viable. The percentage of abnormal spermatozoa was evaluated from the same preparation by examining the morphology of a total count of 100 spermatozoa (Golzar Adabi et al., 2007).

2.3 | Testes weight and testosterone hormone level

The individual live body weight of each bird was recorded weekly from 48 to 64 weeks of age. At the end of the experiment, after killing the birds, the abdominal cavity was opened and testes dissected out. The weight of each testes was measured by using an electronic analytical scale.

At weeks 52 and 64 of the experiment, about 5 h after the beginning of photoperiod, ~11:00 AM, blood samples were obtained from the brachial vein. The blood was centrifuged at 1000 × g for 10 min at 4°C to isolate the plasma. The concentration of the testosterone hormone was measured using a chicken-specific enzyme-linked immunosorbent assay kit (Cat no. MBS9424390, MyBioSource), according to the manufacturer's instruction. This assay was performed in 96-well plates and absorbance was measured at 450 nm.

2.4 | Sperm fatty acid analysis

The fatty acid composition of sperm at the end of the experimental period was analysed according to Folch et al. (1957) and methylated with 5% boron trifluoride methanol complex in methanolic solution. For lipid extraction, chloroform:methanol (2:1 vol/vol) was dissolved in a methanolic NaOH solution (2%). Using *n*-hexane and saturated NaCl solutions, methylated fatty acids were separated and analysed by gas chromatograph equipped with a BPX70 capillary column (SGE capillary column; length, 30 m; I.D., 0.22 mm; film thickness 0.25 μm; 70% cyanopropyl polysilphenylene-siloxane stationary phase), film and flame ionization detector. The gas chromatography conditions were as follows: injector temperature, 240°C; detector temperature, 300°C; carrier gas, helium; split ratio, 1:30; temperature programme, 180°C for 2 min followed by an increase to 230°C at 5°C/min, which was maintained at 24°C. The fatty acid percentage was integrated and then calculated by means of direct normalization of the peak areas. Each fatty acid was identified in the form of a methyl ester by comparing the retention times with the standard acquired from Sigma.

2.5 | Artificial insemination and fertility

Reproductive performance was measured using artificial insemination of hens with fresh sperm. This was achieved following the method of Akhlaghi, Ahangari, Navidshad, et al. (2014) and Akhlaghi, Ahangari, Zhandi, et al. (2014), with a slight modification. Briefly, semen samples in each treatment group were pooled and diluted in Lake's diluent. At 02:00 PM on certain days, 20 hens belonging to each experimental group were inseminated (200×10^6 spermatozoa/hen) on two occasions per week. The eggs were collected over 5 days after the last insemination, were numbered and stored at 13°C and 75% humidity until incubated for fertility assessment and hatchability rates. In each group, 300 settable eggs were set (60 eggs in each weekly set) on incubator trays and disinfected with formaldehyde gas for 15 min according to the recommended concentration (1.2 ml of formalin was added to 0.6 g of potassium permanganate; Asl et al., 2018b). Candling the eggs was performed on 7 days of incubation to assess the fertility rate. Hatching rate was calculated after 21 days of incubation based on the number of fertilized eggs.

2.6 | Lipid peroxidation

With every 2-week intervals from 52 to 64 weeks of age, the pooled semen samples of two birds in each replicate were used for the determination of lipid peroxidation of the semen samples. Thiobarbituric acid reaction was used to measure MDA concentration as an indicator of lipid peroxidation (Esterbauer & Cheeseman, 1990). Briefly, 1 ml of the semen was mixed with 1 ml of 20% (w/v) trichloroacetic acid to sediment precipitate protein. The precipitate was pelleted by centrifuging (960*g* for 15 min) and 1 ml of the supernatant was incubated with 1 ml of 0.67% (w/v) thiobarbituric acid in a water bath at 100°C for 10 min. After cooling, the

absorbance was determined by a spectrophotometer (UV-1200, Shimadzu) at 532 nm and results expressed as nmol/ml.

2.7 | Statistical analysis

All percentage data were subjected to arcsin square root transformation (Steel & Torrie, 1960). All data are presented as least squares means. Single and repeated measurement data were analysed by general linear model and mixed model respectively (SAS Institute, 2001). The model for single-measurement data was: $Y_{ijk} = \mu + FO_i + R_j + (FO \times R)_{ij} + \varepsilon_{jik}$, where Y_{ikj} is any observation, μ is the overall mean, FO_i is the effect of the FO level (0% and 2%), R_j is the effect of the rooibos level (0%, 1.5% and 3.0%), $(FO \times R)_{ij}$ is the interactions, and ε_{ijk} is the residue. The model for repeated measurement data was: $Y_{ijkl} = \mu + FO_i + R_j + T_k + (FO \times R)_{ij} + (FO \times T)_{ik} + (R \times T)_{jk} + (FO \times R \times T)_{ijk} + \varepsilon_{jikl}$, where Y_{ijkl} is any observation, μ is the overall mean, FO_i is the effect of the FO level (0% and 2%), R_j is the effect of the rooibos level (0%, 1.5% and 3.0%), T_k is the effect of sampling time (52, 54, 56, 58, 60, 62 and 64 weeks of age), $(FO \times R)_{ij}$, $(FO \times T)_{ik}$, $(R \times T)_{jk}$ and $(FO \times R \times T)_{ijk}$ are the interactions, and ε_{ijk} is the residue. Statistical differences among various group means were determined by Tukey's test and the values of $p < 0.05$ were considered to be statistically significant.

3 | RESULTS

3.1 | Semen parameters

Table 4 shows the results of the effect of the experimental diets on the semen traits in male broiler breeders. At 52 and 64 weeks of age, and overall period, the semen concentration was significantly ($p < 0.05$) affected by feeding of rooibos. The roosters that received 1.5% and 3% rooibos had higher sperm concentrations when compared to that of the control group ($p < 0.05$). There was no main effect for FO alone or when the FO was combined with rooibos on the semen concentration ($p > 0.05$).

At 52 and 64 weeks of age, the seminal volume was not affected by feeding of FO and rooibos ($p > 0.05$). For the overall period, dietary FO did cause a significant ($p < 0.05$) decrease in the semen volume, whereas roosters receiving the 1.5% and 3% rooibos supplemented diet, had a significantly ($p < 0.05$) higher semen volume. The interactive effect of FO and rooibos on semen volume at Week 64 showed that control birds had the highest semen volume ($p < 0.05$). These parameters for overall period were not significant ($p > 0.05$).

For the overall period, feeding roosters either the FO or the rooibos-supplemented diet alone did not affect the sperm motility significantly ($p > 0.05$), although the combined FO and rooibos diet (refer to the F2R3 group) caused the highest ($p < 0.05$) sperm motility.

Sperm livability was not affected by the inclusion of FO in the diet, but varied significantly depending on the rooibos levels, with an increase when including 1.5% and 3% in the diets at Week 64 ($p < 0.05$). In addition, at Week 64 and overall period, this parameter was affected by the interaction of FO and rooibos ($p < 0.05$). At overall, the highest ($p < 0.05$) percent of livability was recorded in birds receiving the 2% FO and 3% rooibos diet when compared to those in the F2R0 and control groups.

Abnormal sperm percentage was not affected by the feeding of FO nor by the interaction of FO and rooibos ($p > 0.05$). However, dietary rooibos alone, at the level of 1.5%, caused spermatozoa of birds to be the least ($p < 0.05$) abnormal.

3.2 | Body weight and testes weight changes

The effects of dietary treatments on body and testicular weight and testes weight/body weight ratio are shown in Table 5. There was no interaction of dietary FO and rooibos on these parameters ($p > 0.05$). Dietary treatments had no significant effect on the initial and ultimate live body weights of roosters ($p > 0.05$). Dietary FO as main factor had significant effect on testes absolute and relative weights. These parameters were significantly higher in birds fed on diets containing FO ($p < 0.05$). On the other hand, the effect of different levels of dietary rooibos as a main effect on testes weight and relative testes weight/body weight were also significant ($p < 0.05$). So that birds fed on diet containing 3% rooibos had the highest testes weight (28.84 vs. 27.19 g) and relative testes weight/body weight (0.557 vs. 0.526) compared to diet without rooibos ($p < 0.05$).

3.3 | Sperm fatty acids

Table 2 shows the fatty acids composition of experimental diets and FO and Table 6 represents the fatty acid profile of sperm. Trans-fat levels in the diet and sperm of all treatments were very low. FO diets contained greater proportion of EPA (20:5 n-3) and DHA (22:6 n-3), and lower level of linoleic (18:2 n-6). Although FO diet consists of considerable amount of PUFA, the ratio of n-6/n-3 is lower due to the existence of higher levels of n-3 in PUFA composition. In Table 6, it can be clearly seen that this effect is also reflected on fatty acids profile of sperm. So that, for example, when F0R0 and F2R0 are compared, the level of EPA (9.9%) and DHA (44.5%) were high, and the level of n-6 (11.39%) was significantly low in the sperm of birds fed on FO. Consequently, the ratio of n-6/n-3 was 53.73% lower, too.

3.4 | Reproductive performance and testosterone hormone

Fertility and hatchability rates for the weeks of egg collection are summarized in Table 7. Fertility rates were significantly ($p < 0.05$) affected when considering the different groups. The fertility rates were significantly increased in the groups consuming the

TABLE 4 Effects of fish oil and rooibos levels on semen charecteristics in roosters

Treatments	Concentration, 1 × 10^9 spz/ml			Semen volume, ml			Motility, %			Livability, %			Abnormal, %		
	52 wks	64 wks	Overall	52 wks	64 wks	Overall	52 wks	64 wks	Overall	52 wks	64 wks	Overall	52 wks	64 wks	Overall
FO															
0	4.04	3.12	3.69	0.55	0.44	0.49^{a}	86.05^{b}	81.07	83.45	80.8	67	76.51	8.2	15.2	10.88
2	4.27	3.17	3.80	0.53	0.42	0.48^{b}	89.1^{a}	81.94	84.98	81.1	67	76.04	7.9	15.9	10.99
SEM	0.12	0.99	0.72	0.009	0.007	0.003	0.64	0.53	0.35	0.76	0.81	0.37	0.42	0.71	0.3
p	0.19	0.71	0.14	0.30	0.04	<0.0001	0.002	0.67	0.36	0.77	0.48	0.49	0.57	0.48	0.11
Rooibos															
0	3.65^{b}	2.59^{b}	3.15^{b}	0.54	0.44	0.48^{b}	85.6^{b}	81.21	83.04	80.0	65	73.06^{b}	8.0	13.2^{b}	10.94^{b}
1.5	4.46^{a}	3.46^{a}	4.03^{a}	0.53	0.42	0.48^{b}	87.5^{ab}	81.01	83.99	81.0	68	77.99^{a}	8.1	16.1^{ab}	10.83^{c}
3	4.36^{a}	3.42^{a}	4.06^{a}	0.55	0.42	0.49^{a}	89.17^{a}	82.14	85.62	81.9	68	77.77^{a}	8.0	17.2^{a}	11.04^{a}
SEM	0.15	0.12	0.81	0.011	0.009	0.003	0.83	0.67	0.004	0.82	0.11	0.45	0.52	0.84	0.37
p	0.001	<0.0001	<0.0001	0.23	0.24	0.002	0.02	0.43	0.74	0.028	0.10	<0.0001	0.98	0.007	0.008
FO × Rooibos															
F0R0	3.64	2.73	3.27	0.55	0.48^{a}	0.49	85.04	81.18	83.15^{c}	79.0	67^{ab}	74.87^{b}	8.2	13.2	10.56
F0R1.5	4.18	3.40	3.88	0.53	0.41^{b}	0.48	85.41	80.16	82.92^{c}	81.8	68^{ab}	77.99^{a}	8.2	15.0	10.96
F0R3	4.29	3.22	3.93	0.56	0.43^{ab}	0.50	87.15	82.04	84.29^{bc}	81.2	65^{ab}	76.67^{a}	8.2	17.3	11.13
F2R0	3.65	2.38	3.03	0.53	0.40^{b}	0.46	86.16	81.14	82.92^{c}	80.8	63^{b}	71.25^{c}	7.8	13.3	11.32
F2R1.5	4.74	3.51	4.19	0.53	0.43^{ab}	0.49	90.14	81.22	85.06^{ab}	80.1	69^{ab}	78^{a}	8.0	17.2	10.69
F2 R3	4.42	3.61	4.18	0.54	0.42^{ab}	0.48	91.04	81.19	86.96^{a}	82.6	70^{a}	78.87^{a}	7.9	17.0	10.95
SEM	0.21	0.16	0.12	0.016	0.013	0.005	0.92	0.84	0.006	0.16	0.22	0.64	0.71	0.12	0.52
p	0.42	0.09	0.90	0.76	0.0005	0.20	0.31	0.33	0.004	0.35	0.03	0.01	0.96	0.55	0.20

Note: Effects of fish oil and rooibos levels on semen characteristics in roosters. Different letters (a–c) within the same column show significant differences among the groups ($p < 0.05$).

Abbreviations: BW, body weight; F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos; FO, fish oil; wks, weeks.

TABLE 5 Means BW, absolute and relative testes weight from rooster fed diets with the different levels of FO and rooibos

Treatments	Initial live BW (g)	Final live BW (g)	Testes weight (g)	Relative testes weight/BW (g/100 g BW)
FO				
0	4750.5	5167.0	26.83[b]	0.518[b]
2	4750.7	5173.2	29.22[a]	0.566[a]
SEM	3.88	11.5	0.11	0.002
p	0.97	0.70	<0.0001	<0.0001
Rooibos				
0	4750.7	5173.0	27.19[c]	0.526[c]
1.5	4750.6	5172.1	28.04[b]	0.544[b]
3	4750.5	5162.2	28.84[a]	0.557[a]
SEM	4.75	14.1	0.13	0.002
p	0.99	0.91	<0.0001	<0.0001
FO × Rooibos				
F0R0	4750.6	5171.5	25.96	0.501
F0R1.5	4750.7	5169.1	26.85	0.522
F0R3	4750.2	5160.3	27.66	0.532
F2R0	4750.8	5174.4	28.42	0.550
F2R1.5	4750.6	5175.1	29.22	0.566
F2 R3	4750.7	5170.1	30.02	0.582
SEM	6.72	19.9	0.18	0.003
p	0.99	0.98	0.96	0.64

Note: Different letters (a–c) within the same column show significant differences among the groups ($p < 0.05$).

Abbreviations: BW, body weight; F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos; FO, fish oil.

rooibos-supplemented diets, for the 1.5% rooibos the fertility rate was 81.83% and for the 3% rooibos it as 83.67% respectively ($p < 0.05$). Considering the interaction of FO and rooibos, the 2% FO combined with 3% rooibos group had higher fertility rates when compared to the other groups ($p < 0.05$). In contrast, the lowest rate of fertility was observed in the group consuming the 2% FO and 0% rooibos supplemented diet ($p < 0.05$). The percentage of hatching rate based on the number of fertilized eggs was not significantly ($p > 0.05$) affected by the different diets.

The effects of the different diets on plasma testosterone concentration at 52 and 64 weeks of age and the difference between are shown in Table 7. At 52 weeks, the plasma testosterone level decreased ($p < 0.05$) from 2.30 to 2.09 nmol/l with inclusion of 2% dietary FO and the effect of different levels of dietary rooibos were not significant as a main effect during this period. In contrast, at Week 64, roosters fed the 2% FO showed higher ($p < 0.05$) testosterone levels (2.56 vs. 2.06 nmol/l). The effect of dietary rooibos levels was significant during this period so that birds fed on diet containing 1.5% and 3% rooibos had higher ($p < 0.05$) testosterone hormone level compared to roosters with 0% rooibos in the diet. Plasma testosterone was influenced by the interaction between FO and rooibos at 64 weeks. Adding 1.5% and 3% dietary rooibose to a diet containing FO significantly increased ($p < 0.05$) the plasma testosterone level from 1.88 to 2.76 and 3.03 nmol/l respectively.

3.5 | Lipid peroxidation

Results of MDA production as an indicator of lipid peroxidation of sperm have been shown in Table 7. There was no significant ($p > 0.05$) difference in this parameter at 52 weeks. However, at 64 weeks and overall period, supplementation of the diet with 2% FO caused a significant ($p < 0.05$) increase by 28.1% and 39.6% in semen MDA level compared to unsupplemented birds. On the other hand, in the case of supplementing the feed with 1.5% and 3% rooibos, semen MDA significantly lowered to 39.2% and 44.8%, respectively, all through the trial ($p < 0.05$). For the overall period, a significant ($p < 0.05$) interaction between the FO and rooibos was also observed for MDA. The semen MDA level decreased ($p < 0.05$) from 2.73 to 1.56 with increasing rooibos from 0% to 3% at low level of FO (F0R3), whereas inclusion of FO to a diet without containing rooibos significantly ($p < 0.05$) increased the MDA level and adding 1.5% and 3% Rooibos to this diet decreased ($p < 0.05$) the MDA level by 42.7% and 46.1% respectively.

4 | DISCUSSION

This study was designed to investigate the effect of dietary n-3 fatty acids and rooibos (*A. linearis*) on semen quality, sperm fatty acids, and reproductive performance of aged Ross breeder roosters. All the FO fed groups (F2R0, F2R1.5 and F2R3) had the higher amounts of EPA and DHA when compared to the other groups, confirming that dietary n-3 PUFAs were transferred into spermatozoa in these groups (Blesbois et al., 1997). In this study, rooibos, as a source of dietary antioxidants, improved several parameters measured in semen, including testosterone concentration, MDA level, testes weight and reproductive performance of aged roosters. Previous research showed that herbal antioxidants such as dried tomato pomace (Saemi et al., 2012), sage extract (Ommati et al., 2013), dried ginger rhizome (Akhlaghi, Ahangari, Navidshad, et al., 2014) and dried apple pomace (Akhlaghi, Ahangari, Zhandi, et al., 2014) benefitted the male reproduction capacity, especially the sperm quality. In the current study when diets were supplemented with both rooibos and FO, no effects were reported on the sperm concentration, semen volume, abnormality of sperm, body weight and hatchability at the end of experimental period. This may be related to the age of the roosters in the current study (52–64 weeks). Previous studies have

TABLE 6 Fatty acids composition of sperm of rooster fed diets with the different levels of fish oil and fermented rooibos

Fatty acid	Molecular formula	F0R0 control	F0R1.5	F0R3	F2R0	F2R1.5	F2R3	Pooled SEM	*p*
Palmitic	16:0	13.3	13.25	13.75	13.69	12.46	13.01	0.21	0.12
Stearic	18:0	23.22	23.16	23.01	23.08	23.02	23.41	0.33	0.09
Arachidic	20:0	0.84	0.8	0.9	0.93	0.95	1.2	0.09	0.14
Lignoleric	24:0	0.18	0.19	0.18	0.15	0.12	0.14	0.01	0.08
Heptadecenoic	17:1	1.14	1.11	1.17	1.13	1.06	1.03	0.002	0.07
Oleic	18:1 n-9	14.8	14.72	14.64	14.08	14.66	13.29	0.27	0.16
cis-11-eicosenoic	20:1	4.19	4.38	4.2	4.02	4.1	3.43	0.06	0.09
Erucic	22:1 n-9	0.07	0.07	0.05	0.06	0.04	0.06	0.005	0.15
Nervonic	24:1	0.37	0.36	0.35	0.36	0.41	0.38	0.03	0.24
Linoleic	18:2 n-6	5.18^{a}	5.09 ^{a}	5.03^{a}	4.65^{b}	4.78^{b}	4.62^{b}	0.11	0.02
cis-11.14 eicosadienoic	20:2 n-6	1.85	1.82	1.73	1.65	1.34	1.52	0.13	0.15
cis-8.11.14. eicosatrienoic	20:3 n-6	2.69	2.76	2.73	2.58	2.16	2.03	0.16	0.08
Arachidonic	20:4 n-6	10.62^{a}	10.47^{a}	9.88^{a}	8.79^{b}	9.1^{b}	8.93 ^{b}	0.19	0.01
DTA	22:4 n-6	19.26	19.25	19.94	19.89	19.68	19.44	0.22	0.13
Linolenic	18:3 n-3	0.91	0.98	0.94	1.01	1.05	1.02	0.07	0.09
EPA	20:5 n-3	0.61^{b}	0.74^{b}	0.65^{b}	1.1^{a}	1.32^{a}	1.41^{a}	0.11	0.003
DHA	22:6 n-3	1.01^{b}	1.05^{b}	1.08^{b}	3.08^{a}	4.03^{a}	4.89^{a}	0.18	0.009
Others		0.13	0.16	0.12	0.11	0.13	0.19	***	***
ΣSFA		37.54	37.4	37.84	37.85	36.55	37.76	0.42	0.21
ΣMUFA		20.2^{a}	20.28^{a}	20.06^{a}	19.29^{b}	19.86^{b}	18.19^{b}	0.29	0.02
ΣPUFA		42.13	42.16	41.98	42.75	43.46	43.86	0.56	0.14
n-6		39.6^{a}	39.39^{a}	39.31^{a}	37.56^{b}	37.06^{b}	36.54^{b}	0.41	0.009
n-3		2.53^{b}	2.77^{b}	2.67^{b}	5.19^{a}	6.4^{a}	7.32^{a}	0.13	0.002
n-6/n-3		15.65^{a}	14.22^{a}	14.72^{a}	7.24^{b}	5.79^{b}	4.99^{b}	0.11	0.007

Note: Different letters (a–b) within the same column show significant differences among the groups ($p < 0.05$).

Abbreviations: ΣMUFA, total monounsaturated fatty acids; ΣPUFA, total polyunsaturated fatty acids; ΣSFA, total saturated fatty acids; DHA, docosahexaenoic acid; DTA, docosatetraenoic; EPA, eicosapentaenoic acid; F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos.

reported on decreased volume and concentration of semen in aging male broiler breeders (Kelso et al., 1997). In this regard, it has been reported that dietary PUFAs could not prevent the reduction of sperm concentration in the aged broiler breeder (Akhlaghi, Ahangari, Navidshad, et al., 2014). In another study using an aqueous rooibos extract as drinking water for rats, Opuwari and Monsees (2014) did not find significant differences in sperm kinetic parameters, although sperm concentration, motility and viability showed higher values than those of the control group (Awoniyi et al., 2012; Opuwari & Monsees, 2014). Our results provide further support to the positive effects of rooibos on motility and livability of sperm. The reason for the improvement of sperm viability is probably related to the antioxidant defence system of semen (Asl et al., 2018b). This finding is in concert with Raei et al. (2021), who reported that the use of camphor as antioxidant source in rooster's diet improved some sperm quality parameters. The antioxidant capacity as a defence mechanism against lipid peroxidation in semen is important in maintaining sperm livability (Raei et al., 2021; Surai et al., 2011). On the other hand, the presence of a high amount of PUFAs in the plasma membrane of rooster sperm rendered the sperm membrane more susceptible to oxidation (Feyzi et al., 2018). Thus, the roosters that received FO alone had a lesser livability of sperm than the roosters that received FO combined with rooibos. Although previously reported dietary PUFA supplementation can improve the sperm membrane (Asl et al., 2018b), we did not find this result. It seems that the antioxidant effect of rooibos administration in the current study was responsible for the increased sperm motility and livability. Contrary to our results, Superchi et al. (2005) reported an enhancement in sperm

TABLE 7 Means of semen parameters from rooster fed diets with the different levels of FO and rooibos

Treatments	Fertility (%)	Hatchability (%)	MDA (nmol/ml)			Testosterone (nmol/l)		
			52 wks	64 wks	Overall	52 wks	64 wks	Difference
FO								
0	78.67	80.89	2.34	1.92^{b}	2.02^{b}	2.30^{a}	2.06^{b}	0.25^{a}
2	81.33	81.91	2.52	2.46^{a}	2.82^{a}	2.09^{b}	2.56^{a}	-0.47^{b}
SEM	1.02	1.10	0.16	0.15	0.14	0.07	0.06	0.10
p	0.08	0.52	0.45	0.02	0.03	0.04	<0.0001	<0.0001
Rooibos								
0	74.50^{b}	79.25	2.40	3.93^{a}	2.99^{a}	2.16	1.82^{b}	0.34^{a}
1.5	81.83^{a}	82.37	2.39	1.26^{b}	1.82^{b}	2.19	2.50^{a}	-0.32^{b}
3	83.67^{a}	82.58	2.50	1.40^{b}	1.65^{b}	2.24	2.60^{a}	-0.36^{b}
SEM	1.25	1.35	0.20	0.19	0.17	0.09	0.07	0.12
p	<0.0001	0.17	0.90	<0.0001	<0.0001	0.79	<0.0001	0.0002
FO × Rooibos								
F0R0	75.67^{bc}	79.33	2.23	3.61	2.73^{a}	2.28	1.75^{c}	0.53
F0R1.5	79.67^{abc}	80.69	2.22	1.07	1.78^{bc}	2.27	2.25^{bc}	0.02
F0R3	80.67^{abc}	82.66	2.58	1.11	1.56^{c}	2.36	2.17^{c}	0.2
F2R0	73.33^{c}	79.17	2.57	4.25	3.25^{a}	2.04	1.88^{c}	0.16
F2R1.5	84.00^{ab}	84.06	2.56	1.44	1.86^{b}	2.10	2.76^{ab}	−0.65
F2 R3	86.67^{a}	82.50	2.43	1.70	1.75^{bc}	2.12	3.03^{a}	−0.91
SEM	1.76	1.92	0.28	0.26	0.24	0.12	0.10	0.17
p	0.0002	0.58	0.62	0.85	0.029	0.93	0.0029	0.10

Note: Different letters (a–c) within the same column show significant differences among the groups ($p < 0.05$).

Abbreviations: BW, body weight; F0R0, control diet with no rooibos and/or fish oil; F0R1.5, containing only 1.5% rooibos; F0R3, containing only 3% rooibos; F2R0, containing only 2% fish oil; F2R1.5, containing 2% fish oil and 1.5% rooibos; F2R3, containing 2% fish oil and 3% rooibos; MDA, malondialdehyde; wks, weeks.

concentration and percentage of live sperm in boars receiving rosemary oil extract, although the sperm motility and percentage of abnormal sperms were not affected. Also, recent studies showed that supplementation with antioxidants such as the catalase and vitamin E (Moghbeli et al., 2016), hyaluronic acid (Lotfi et al., 2017) and L-carnitine (Fattah et al., 2017) significantly improved the sperm viability. In human studies, it is reported that motility of spermatozoa depends on the integrity of the mitochondrial sheath, of which phospholipids are a major component. If fatty acids in these phospholipids are oxidized by ROS, spermatozoa will be damaged (Alvarez & Storey, 1982). In addition, Griveau et al. (1995) have also shown that ROS cause a decrease in sperm motility along with an increase in lipid peroxidation. Sperm in vitro senescence and loss of motility is one of the biochemical consequences of lipid peroxidation (Surai et al., 2001). Based on the results of MDA content and motility in the sperm of roosters fed rooibos, it can be concluded that these parameters have a negative correlation with each other (Raei et al., 2021).

In the current study, the relative weight of the testes was significantly changed by the main effect of FO and rooibos levels ($p < 0.05$); however, the interaction of FO and rooibos had no significant effect on the relative weight of the testes ($p > 0.05$). This finding is partially in agreement with Opuwari and Monsees (2014) who reported increased testes weights when male rats consumed rooibos as part of their daily diet. It is well known that the number of spermatozoa is dependent upon the testes weight (Danikowski et al., 2002).

Higher concentrations of PUFAs, especially EPA and DHA in the fatty acid profile of sperm in the F2R0, F2R1.5 and F2R3 groups revealed that dietary n-3 PUFA were successfully transferred into spermatozoa. Increasing the DPA (decosapantaenoic acid), EPA and DHA in the plasma membrane of sperm is very important to improve fertility (Asl et al., 2018a). This positive correlation between n-3 fatty acids in the sperm membrane and motility of sperm were previously reported in other animals (Asl et al., 2018b). However, it is very important point that exorbitance membrane fluidity can damage the plasma membrane leading to reduce cells signal transduction (Esmaeili et al., 2014) that can have detrimental effects on the sperm (Asl et al., 2018b). Similar to our results, Zanussi et al. (2019) reported that aged roosters fed 2% of dietary flaxseed oil, had higher DPA and

DHA (decosahexaenoic acid) when compared to the control group. In a previous study, a decline in the proportion of DHA in sperm with aging was suggested (Cerolini et al., 1997). Recently, Asl et al. (2018b) showed that this can be compensated by supplementation with dietary FO, which significantly increases DHA and total n-3 fatty acids content of spermatozoa.

The FO and rooibos interaction when considering lipid peroxidation (as measured by MDA in the current study) indicated that at least 10 weeks is required to initiate a response. In the current study, the control diet and FO diet supplemented with rooibos exhibited a lower concentration of MDA levels when compared to the diets without rooibos. These finding show that FO needs to be 'protected' against lipid peroxidation and the PUFAs of FO without any protection can cause damage to sperm. In this regard, Awoniyi et al. (2011) found that aqueous extract of fermented rooibos, administered in drinking water significantly decreased the lipid peroxidation levels in the testes of oxidative stress-induced male rats. In a similar study in rat epididymal sperm, the catalase activity was enhanced in rats consuming the rooibos aqueous extract (Awoniyi et al., 2012). In addition, previous studies have shown that rooibos reduced the accumulation of age-related lipid peroxides in the brain of rats consuming this herbal tea for 21 months and inhibited MDA formation in rats' tissues and liver microsomal preparations (Marnewick et al., 2005; Ulicna et al., 2006). Rooibos is an important source of antioxidants due to its rich and unique flavonoid content with numerous studies reporting on its health benefits (Ajuwon et al., 2013). The ability of rooibos to protect against lipid peroxidation may involve one or more of several different antioxidant properties exhibited by rooibos. The protective effect may be due to the ability of the rooibos phenolic constituents not only to bind lipid peroxides, but also their ability to inhibit the lipid peroxidation cascade, either by acting as a sacrificial antioxidant or as a chelator of transition metals that promote lipid peroxidation (Awoniyi et al., 2012; Halliwell & Gutteridge, 2007; Nijveldt et al., 2001). The protective effect may also be associated with the the inhibition of cytochrome P450 metabolism that initiates the lipid peroxidation (Ajuwon et al., 2013).

In the present study, we showed that the concentration of testosterone positively related to the motility and livability of sperm. Spermatogenesis and steroidogenesis in the avian testes are dependent on luteinizing hormone (LH) and follicle-stimulating hormone (FSH), and testosterone (Vizcarra et al., 2010). FSH and LH regulate spermatogenesis via cyclic adenosine 3', 5'-monophosphate (Raei et al., 2021). LH binds to receptors in the membrane of Leydig cells, and stimulates the secretion of testosterone. Testosterone levels determine the testicular development and behaviour of roosters (Feng et al., 2015), while it may also act on the Sertoli and peritubular cells of the seminiferous tubules and stimulate spermatogenesis (O'Donnell et al., 1994). Corresponding to the livability and motility of sperm data, the results of testosterone levels showed that the rooibos-treated groups had higher testosterone levels than the other groups. Contrary to our results, Asl et al. (2018b) reported that diets supplemented with PUFAs increased testosterone levels in breeder roosters. In this study, the reason for the lack of increased testosterone in the FO groups is not clear, but oxidation of unsaturated fatty acids in FO may cause induced oxidative damage in related organs. In fact, the groups that received the rooibos combined with the FO had higher testosterone levels. Therefore, it is proposed that the rooibos protects against oxidation of FO due to its bio-activities that include antioxidant capacity. Similarly, Noh et al. (2012) noticed a significant increase in serum testosterone in aging rats (18 weeks old) after treatment with a mixture of rooibos and dandelion extract, known for its antioxidant capacity.

One of the most important findings in the current study is the increased fertility percentage in roosters fed the diet containing FO combined with 3% rooibos. The fatty acid composition and resistance to oxidation of spermatozoa may be an important predictor of fertility as the hens received the same dose of spermatozoa during artificial insemination (Akhlaghi, Ahangari, Navidshad, et al., 2014; Akhlaghi, Ahangari, Zhandi, et al., 2014; Cerolini et al., 1997). Various studies have shown the effect of different antioxidant feed additives on increased antioxidant capacity of seminal plasma (Akhlaghi, Ahangari, Navidshad, et al., 2014; Asl et al., 2018b; Golzar Adabi et al., 2011). Fertility evaluation using artificial insemination is a very important assessment to confirm the obtained in vitro results (Feyzi et al., 2018; Shahverdi et al., 2015). In other studies, a high correlation was shown between sperm parameters and reproductive performance in avian models (Akhlaghi, Ahangari, Zhandi, et al., 2014; Fattah et al., 2017). This is likewise supported by a report by Opuwari and Monsees (2014), which showed that reproductive functions were improved in male rat fed rooibos. We used rooibos in our study, because it could offer a measure of protection against induced oxidative damage by enhancing the endogenous antioxidant defence mechanisms and thereby improving the sperm quality and function (Awoniyi et al., 2012). As a result, using a proper antioxidant is a good choice for preventing and/or decreasing oxidation effect of n-3 PUFAs.

5 | CONCLUSION

Due to the influence of the fertility of roosters on the broiler breeder flocks, it is necessary to improve their reproductive performance through various approaches, including the feeding strategy. In summary, dietary treatment of aged roosters with rooibos and FO increased semen quality including sperm motility and livability with a decreased level of sperm MDA. Although the results of using FO did not meet our expectations, dietary rooibos supplementation is effective to enhance the antioxidant status of sperm. Based on the results of the current study, dietary rooibos supplementation, recommended at 3% (w/w) can be considered as an influential factor in improving fertility by enhancing the antioxidant status and/or modulating the oxidative distress.

ACKNOWLEDGMENT

This project was funded by MAKT. The authors would like to acknowledge Vice-Chancellor in Research Affairs of Tarbiat Modares University.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

AUTHOR CONTRIBUTIONS

Shahram Golzar Adabi and Mohammad Amir Karimi Torshizi designed the trial. Mohammad Amir Karimi Torshizi submitted the ethics clearance application. Mohammad Amir Karimi Torshizi and Hamid Raei executed the trial. Jeanine L. Marnewick ran the analysis of active substances in Rooibos. Shahram Golzar Adabi did the statistical analyses and interpreted the results. Hamid Raei wrote the draft paper. All authors checked the results and contributed to preparing the manuscript.

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How to cite this article: Golzar Adabi, S., Karimi Torshizi, M. A., Raei, H., & Marnewick, J. L. (2023). Effect of dietary n-3 fatty acid and rooibos (*Aspalathus linearis*) supplementation on semen quality, sperm fatty acids and reproductive performance of aged male broiler breeders. *Journal of Animal Physiology and Animal Nutrition*, 107, 248–261. https://doi.org/10.1111/jpn.13705