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Barley sprouts and D-Aspartic acid supplementation improves fertility, hatchability, and semen quality in aging male broiler breeders by up-regulating StAR and P450$_{SCC}$ gene expressions

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ABSTRACT At 50 wk of age, broiler breeder roosters exhibit a significant decline of fertility. Therefore, the aim of this study was to assess the impact of incorporating barley sprout (**BS**) powder, D-aspartic acid (**DA**), or their combination into the diet on fertility, hatchability, semen quality, and the relative expression of *StAR* and *P450$_{SCC}$* genes in aging broiler roosters. Aging (50 wk) male broiler breeders (n=32) were randomly assigned to one of four dietary treatments (2 × 2 factorial) with 2 levels of BS (0 or 2% basal diet) and DA (0 or 200 mg/kg/BW) for 12 wk. Roosters were individually housed under a 14-h light and 10-h dark cycle, with 150 g/d feed allocation and free access to fresh water, then euthanized. Throughout the study, the body weight of the broiler breeders was measured, along with various parameters related to semen quality, on a weekly basis. Additionally, artificial insemination was performed during the last 2 wk to evaluate reproductive endpoints. The results revealed that both BS and DA decreased ($P < 0.01$) body weight. Interestingly, the inclusion of BS, either alone or in combination with DA, resulted in a significant increase in total and forward sperm motility. Furthermore, it was demonstrated that the seminal concentration of malondialdehyde, a marker of oxidative stress, was significantly decreased by more than 20% in all groups compared to the control. The combination of both BS and DA led to the highest levels of circulating testosterone, as well as the functionality and membrane integrity of sperms. Additionally, it resulted in increased sperm concentrations, production, and penetration, ultimately leading to improved fertility rate and hatchability percentage. Moreover, a positive association between total motility and fertility was observed ($P < 0.01$). Furthermore, the combined supplementation of BS and DA up-regulated the relative mRNA expression of *P450scc* and *StAR* ($P < 0.01$). To summarize, dietary inclusion of BS, DA, or their combination have a potential to improve various aspects of reproductive performance in aging roosters.

Key words: elderly rooster, barley sprout, d-aspartic acid, reproductive performance

2024 Poultry Science 103:103664
https://doi.org/10.1016/j.psj.2024.103664

INTRODUCTION

As roosters grow older, their testicular antioxidant systems become compromised, leading to reproductive dysfunctions caused by testicular apoptosis, impaired spermiation, and changes in serum biochemistry. The decline in reproductive function among aged roosters can be attributed to various factors, such as elevated body weight and oxidative stress, reduced concentrations of reproductive hormones, decreased sperm production and quality, and a decline in antioxidant status (Gao et al., 2021; Nanwei et al., 2021).

There is evidence suggesting that the activities of specific anti-oxidant enzymes decrease as individuals age, which worsens conditions of oxidative stress. For instance, Huang et al. (2018) observed that the aging process is accompanied by a decrease in the expression of crucial anti-oxidants in leydig cells, resulting in excessive oxidative stress and heightened lipid peroxidation. Aging also diminishes the functional expression of steroidogenic enzymes in leydig cells, such as steroidogenic

Received January 14, 2024.
Accepted March 11, 2024.

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acute regulatory protein (***StAR***), cholesterol side-chain cleavage enzyme (***P450scc***), superoxide dismutase (***SOD***), and glutathione peroxidase (***GSH-P$_X$***) (Luo et al., 2001; Huang et al., 2018).

Barley sprouts (**BS**) (*Hordeum vulgare* L.) have garnered attention as a functional food due to their antioxidant and anti-inflammatory effects, as highlighted by Seo et al. (2013) and Eun et al. (2016), respectively. The antioxidant properties of barley sprouts can be attributed to the presence of phenolic compounds and bioactive flavonoids, which are produced during the sprouting process and can be extracted through steeping (Seo et al., 2013). Furthermore, studies have shown that barley sprouts have the potential to improve blood chemistry (Byun et al., 2015), and exhibit beneficial effects on reproductive performance in Japanese quail (Abouelezz et al., 2019) and rats (Khattab et al., 2022).

D-Aspartic acid (**DA**) is a neurotransmitter known for its positive impact on reproductive functions. It has been found to increase the levels of follicle stimulating hormone, luteinizing hormone, and testosterone in the bloodstream. These effects are believed to be a result of its influence on the hypothalamic-pituitary-gonadal axis in mammals (Furuchi and Homma, 2005), pigs (Di Fiore et al., 2014), and rats (D'Aniello et al., 2000). Additionally, DA has been shown to enhance the production of steroidogenic acute regulatory protein (***StAR***) mRNA in rats (Raucci et al., 2014). Studies have revealed high concentrations of DA in various reproductive organs and cells, such as the testicular veins, epididymis, testicular cells, and sperm in rats (Santillo et al, 2014; Santillo et al., 2023). In human studies, DA supplementation has been found to improve sperm concentration, motility (both forward and total), and testosterone levels (D'Aniello et al., 2012). Furthermore, in aging roosters, oral administration of DA has been shown to enhance sperm quality, fertility, and hatchability by activating steroidogenesis pathways (Ansari et al., 2018). Based on these findings, we conducted an experiment to investigate whether dietary supplementation of BS and DA could enhance the reproductive performance of aging roosters.

MATERIALS AND METHODS

Ethical Animal Use and Chemicals

This study was approved by the Animal Care Committee of the Gorgan University of Agricultural Sciences and Natural Resources, Gorgan, Golestan, Iran (Protocol No; 1398/165943/388). All chemicals used in this study were provided by Merck (Darmstadt, Germany) and Sigma-Aldrich (St. Louis, MO), unless otherwise stated.

Sprout Preparation and Extraction

The sprouting procedure was carried out according to the method described by Eun et al. (2016) with slight modifications. Barley seeds (Hordeum vulgare L.) were thoroughly washed and soaked in darkness at 25°C for 24 h, with the water being changed every 2 h. Subsequently, the soaked seeds were allowed to sprout in the dark for 2 d at a temperature range of 22 to 25°C. Once approximately 90% germinated, the seeds were spread on filter paper and placed in a dark environment with a relative humidity of 60% and a temperature range of 22 to 24°C for a duration of 10 d. The sprouts were then collected and dried in a 40°C dryer until they reached a constant water content of 10%. The dried samples were carefully packaged in sealed bags and stored at -20°C until further analysis.

The method for extracting sprouted barley was carried out according to the procedure outlined by Stanković (2011), with certain modifications. Initially, 10 grams of sprouts were macerated for a duration of 10 min using a pestle and mortar. Subsequently, a 10% (weight/volume) extract was prepared by utilizing 70% methanol, which was then stirred for 24 h at room temperature and subsequently filtered. To dry the filtrate extract, a rotatable evaporator was employed at a temperature of 40°C. The resulting dried extract was dissolved in 95% methanol to create a stock solution, which was stored at 4°C until it was used for chemical analysis.

Determination of Total Phenolic Content and Antioxidant Activity of Sprouted Barley

Total phenolic content (**TPC**) was quantified using the Folin-Ciocalteu colorimetric assay with minor modifications (Singleton et al., 1999). A mixture comprising 0.5 mL of the extract and 2 mL of the F-C reagent was prepared. After a 5-min incubation, 1.6 mL of Na_2CO_3 (7.5%, w/v) was added and the solution was further incubated at 45°C for 45 min. To determine the absorbance at 760 nm against a blank solution, a UV/visible spectrophotometer (UV-visible, UV1800, Jinghua Instrument Co., Ltd., Shanghai, China) was utilized. The blank solution consisted of 0.5 mL of 95% methanol, 2.5 mL of 10% F-C reagent, and 2.5 mL of 7.5% Na_2CO_3. Each sample was measured in triplicate, and the resulting values were expressed as milligrams of chlorogenic acid equivalents (**CAE**) per gram of dried extract. The TPC was determined using a calibrated curve ($R2 = 0.991$), with gallic acid as the standard (range, 0.001−0.006 mg/mL).

The conventional 2,2-diphenyl-1-picrylhydrazyl (**DPPH**) free radical scavenging assay was employed to measure the antioxidant activity of barley sprouted methanolic extract (Brand-Williams et al., 1995). The absorbance of the extract was determined at 515 nm using a spectrophotometer. The scavenging activity of the extract was calculated using the following formula:

DPPA scavenging activity % = [(Ac − As sample)/Ac blank] × 100 where Ac and As represent the absorbance of the control and sample, respectively. The antioxidant activity of the extract was quantified as half-maximal inhibitory concentration (**IC_{50}**), which denotes the concentration of the sample required to scavenge

50% of the DPPH free radical. The IC_{50} values were determined through non-linear regression analysis of the scavenging activity of DPPH radicals at various sample concentrations. These IC_{50} values were reported as mg or Trolox equivalent (**TE**) sample (DM)/mL solution of DPPH (Djordjevic et al., 2011).

Roosters and Experimental Design

Thirty-two Hubbard M77 broiler breeder roosters (50 wk of age) were randomly allotted to 4 treatment groups (n = 8 birds/group) in a 2 × 2 factorial arrangement, with factors being BS (0 and 2% of basal diet) and DA (0 and 200 mg/kg BW) for 12 wk. The doses used were based on reports by Abouelezz et al., 2019 (BS) and Ansari et al., 2018 (DA). Roosters were housed in individual cages (60 × 50 × 75 cm), with a 14L:10D photoperiod cycle and 21 to 24°C temperature. Rosters received 150 g per day a standard diet (Table 1) and had free access to fresh water. Every week, the weight of each rooster was measured. Throughout the initial 10 wk of the experiment, the collection and evaluation of semen were conducted on a weekly basis.

Semen Quality

Semen was obtained from roosters using abdominal massage, as described by Burrows and Quinn (1937). A graduated collecting tube was employed to quantify the volume of semen volume, while a Neubauer hemocytometer was utilized to ascertain the sperm concentration. Total sperm production (total sperm per ejaculate × 10^9) was calculated by multiplying ejaculate volume and sperm concentration.

In order to evaluate the total and forward motility of sperm, a diluted pooled semen sample (1:20 in 2.9% sodium citrate) was prepared. A volume of 10 μL of this diluted semen was then placed on a pre-warmed microscope slide at a temperature of 37°C. The sperm motility was subjectively estimated by directly observing the sample under phase-contrast at a magnification of 400 × using a Carl Zeiss microscope (Germany). The evaluation involved assessing the motility of at least 200 sperm per slide. The total motility was expressed as a percentage of motile sperm, while the forward motility was expressed as a percentage of sperm exhibiting moderate or quick movement in a forward direction (Santiago-Moreno et al., 2009; Akhlaghi et al., 2014).

The Eosin-nigrosin staining was employed in order to evaluate the viability and abnormalities of sperm. Sperm that were not stained were classified as live, while those exhibiting any form of morphological abnormality such as detached heads, abaxial heads, malformed heads, bent tails, coiled tails, double tails, or protoplasmic droplets were considered abnormal. In order to assess the morphology, a mixture of semen and stain was applied onto a warm slide and then spread onto a second slide. Viability was determined by examining 200 sperm under phase-contrast at a magnification of 1,000 × . Sperm that displayed partial or complete purple staining were regarded as dead, whereas those without any staining were deemed viable. The percentages of viable and abnormal sperm were calculated based on the total number of sperm evaluated. (Lukaszewicz et al., 2008; Fattah et al., 2017a).

In order to assess the functionality of the sperm plasma membrane, a hypo-osmotic swelling test (**HOST**) was carried out with slight adjustments (Santiago-Moreno et al., 2009). The test involved the combination of 10 μL of pooled semen with 200 μL of a hypoosmotic sodium citrate solution (100 mOsm). After a 30-min incubation period, the membrane integrity of the sperm was observed using a phase-contrast microscope (Carl Zeiss, Germany). A total of 200 sperm, with either swollen or non-swollen tails, were identified and categorized as having either intact or compromised membrane integrity, respectively.

Table 1. Ingredients and chemical composition of the standard diet fed to broiler breeder roosters.

Ingredients	Content (%)
Corn grain	62.1
Soybean meal	11.2
Wheat barn	22.97
Dicalcium phosphate	0.87
Calcium carbonate	1.55
Sodium chloride	0.22
DL-Met	0.09
L-Lys	0.1
Soybean Oil	0.3
Vitamin premix[1]	0.3
Mineral premix[2]	0.3
Calculated composition	
Digestible amino acids	
Lysine	0.5
Methionine	0.3
Leucine	0.53
Methionine and cystine	0.5
Tryptophan	0.12
Arginine	0.65
Isoleucine	0.42
Valine	0.5
Threonine	0.39
ME(Kcal/Kg)	2650
CP	12.5
Ca	0.9
Available P	0.4
Na	0.17
Cl	0.16
K	0.6

[1]Supplied per kilogram of diet: vitamin A, 15000 IU; vitamin E, 100 IU; vitamin K_3, 5 mg; vitamin B_{12}, 0.03 mg; vitamin D, 3000 IU; riboflavin, 10 mg; niacin, 60 mg; pantothenic acid, 15 mg; pyridoxine, 5 mg; biotin, 0.2 mg; folic acid, 2 mg.

[2]Supplied per kilogram of diet: Fe, 60 mg; Mn, 60 mg; Zn, 100 mg; I, 2 mg; Se, 0.4 mg.

Reproductive Performance

In order to assess the reproductive performance, artificial insemination (**AI**) was conducted during the 11th (first and second d) and 12th (first d) weeks of the experiment. A total of 120 broiler breeder hens (n = 30/group) belonging to the same strain and having no prior exposure to male birds for a duration of 1 mo were accommodated in floor pens layered with wood shavings.

Table 2. Ingredients and chemical composition of the standard diet fed to broiler breeder hens.

Ingredients	Content (%)
Corn grain	63.3
Soybean meal	21
Wheat barn	5.2
Dicalcium phosphate	1.1
Calcium carbonate	7.5
Sodium chloride	0.25
DL-Met	0.15
Soybean Oil	1
Vitamin premix[1]	0.25
Mineral premix[2]	0.25
Calculated composition	
Digestible amino acids	
Lysine	0.68
Methionine	0.29
Methionine and cystine	0.56
Tryptophan	0.18
Threonine	0.55
ME(Kcal/Kg)	2750
CP	17
Ca	3.1
Available P	0.4
Na	0.16
Cl	0.17
K	0.7

[1]Supplied per kilogram of diet: vitamin A, 15,000 IU; vitamin E, 100 IU; vitamin K_3, 5 mg; vitamin B_{12}, 0.03 mg; vitamin D, 3,000 IU; riboflavin, 10 mg; niacin, 60 mg; pantothenic acid, 15 mg; pyridoxine, 5 mg; biotin, 0.2 mg; folic acid, 2 mg.

[2]Supplied per kilogram of diet: Fe, 60 mg; Mn, 60 mg; Zn, 100 mg; I, 2 mg; Se, 0.4 mg.

The hens were subjected to a light and dark cycle of 14 h and 10 h, respectively, and were provided with a standard diet (Table 2). This diet consisted of 17% crude protein, 2750 kcal/kg diet metabolizable energy, 3.1% calcium, and 0.4% available phosphorus.

In order to address the issue of low fertility caused by advanced age, hens with high production rates (35 wk old with approximately 90% egg production) were selected for the study. AI was performed using semen obtained from roosters in each treatment group (8 roosters per treatment). The semen was pooled and diluted in a modified Beltsville extender, as described by Ansari et al. (2017). Subsequently, 30 individual hens from each treatment group were inseminated with the pooled semen, with a dosage of 100×10^6 sperm per hen, twice a week for a duration of 2 wk. Starting from 2 d after the first AI, eggs were collected daily for a period of 10 consecutive d. These eggs were then stored at a temperature of 13°C and a humidity level of 75% until they were ready for incubation. A total of 802 settable eggs were collected for the study, with 191, 202, 195, and 214 eggs allocated to the Control, BS (barley sprout, 2% basal diet), DA (D-aspartic acid, 200 mg/kg/BW), and BS +DA treatment groups, respectively. These eggs were fumigated and subsequently incubated for a duration of 18 d at a temperature of 37.7°C. After the incubation period, the eggs were transferred to the hatcher until d 21. At the end of the incubation period, the fertility rate (calculated as the number of fertile eggs divided by the total number of eggs set) and the hatchability rate (calculated as the number of chicks divided by the number of fertile eggs) were determined (Akhlaghi et al., 2014).

Sperm Penetration Assay

The perivitelline layer (**PVL**) sperm penetration assay is commonly utilized to assess overall fertility and sperm performance (Bramwell et al., 1996). A higher number of sperm holes in the PVL indicates greater fertility. To conduct this procedure, albumin is separated from the yolks, and any excess albumin and chalaziferous residual are eliminated from the PVL by washing the yolks in a 1% NaCl solution for 30 min. Subsequently, 2% NaCl is added to moisten the yolk, and a 1-cm^2 portion of the PVL that covers the germinal disc is carefully removed and vigorously shaken in a 1% NaCl solution to eliminate any adhering yolk material. Each section is then meticulously positioned on a slide to prevent wrinkles and overlapping. The slides are fixed using 15% formalin, stained with Schiff's reagent, and air-dried. The number of holes per square millimeter overlying the germinal disc is subsequently counted using light microscopy (Carl Zeiss, German; 400 × magnification).

Lipid Peroxidation and Testosterone

The concentration of Malondialdehyde (**MDA**) in semen, which serves as an indicator of lipid peroxidation, was assessed using the thiobarbituric acid reaction method (Esterbauer and Cheeseman, 1990). The quantification of thiobarbituric acid reactive substances involved comparing the absorption with a standard curve of MDA equivalents, which were generated through the acid-catalyzed hydrolysis of 1,1,3,3-tetramethoxypropane. The absorbance was measured at 532 nm using a spectrophotometer (UV-1200, Shimadzu, Japan), and the MDA concentrations were expressed in nmol/mL.

At the end of study, blood samples were obtained from the brachial vein using a vacuum blood collection tube, which were then centrifuged at 1,500 × *g* for 15 min. The resulting serum was separated and stored at a temperature of -20°C. The measurement of testosterone was carried out using an ELISA kit (Monobind Inc., Costa Mesa, CA). The assays were performed in 96-well plates, and the absorbance was determined at a wavelength of 450 nm. The intra-assay coefficients of variation for the testosterone assay were found to be 6.08%, while the sensitivity of the assay was determined to be 0.0576 ng/mL.

Real-time Expression of Candidate Genes

The relative expression of 2 candidate genes including *StAR* and *P450scc* was measured in testes samples using real-time quantitative PCR. Samples of right testes were frozen in liquid nitrogen to prevent RNA degradation and subjected to total RNA extraction and real-time polymerase chain reaction (**RT-PCR**). Engineered primers, used in the RT-PCR were designed using Primer 3 software, for *StAR* and *P450scc* with *β-actin* as internal control (Table 4). Total RNA was extracted using the RNeasy Mini Kit (Cat. No. 74104; Qiagen)

according to the manufacturer's protocol. Using the QuantiTec Reverse Transcription Kit (Cat. No. 205311; Qiagen, GmbH, Germany), cDNA was synthesized with the reaction conditions of 42.8°C for 30 min and 95.8 °C for 3 min. Quantification of all transcripts was performed using QuantiFast SYBR Green PCR Kit (Cat. No. 204054; Qiagen, GmbH, Germany) in a 20 μL reaction volume containing 1 μL single-strand cDNA, 10 μL of master mix, 0.5 μL of either forward and reverse primers and 8 μL of distilled H_2O in 20 μL by a Rotor-Gene 6000 Real-Time PCR software (Corbett Research, Sydney, Australia). The PCR program consisted of a 5 min activation step at 95°C, followed by 40 cycles of 15 s at 95°C, and 40 s at 60°C. At the end of each PCR, for all genes, a melting curve analysis was performed at 0.1°C/s to verify product specificity. The efficiency of the tests was > 95% and the standard curve R^2 was > 0.999. The qPCR data were analyzed by the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001).

Statistical Analysis

Data were analyzed in a 2 × 2 factorial arrangement for dietary treatments, with ANOVA used to determine main effects and the interaction. The Shapiro-Wilk test was used to assess data normality. Arc sine test was used for transformation of percentage data when appropriate. Single and repeated-measurement data were analyzed by PROC GLM and PROC MIXED, respectively (SAS, 2002). Rooster's body weight was included as a covariate for analysis of variance. Fertility and hatchability rates were analyzed using GENMOD. Coefficient correlations determined using Pearson 2 tailed analysis. If there was a significant ($P < 0.05$) main effect or interaction, differences between groups were determined using Duncan's Test.

RESULTS

Total Phenolic Content and Antioxidant Activity

TPC and antioxidant function of the methanol extract of sprouted barley are shown (Table 3). The Folin-Ciocalteu procedure was employed to determine the TPC in sprouted barley, with gallic acid serving as the standard. A calibration curve was constructed using absorbance values obtained from different concentrations of gallic acid. The TPC was then quantified as milligrams of gallic acid equivalents (**GAE**) per gram of dry weight (mg/g) per sample. Consequently, the TPC value of the sprouted barley extract was found to be 302.8 ± 10.9 mg GAE/g. Sprouted barley exhibited a rich presence of phenolic compounds, with vanillic acid, catechin, and epicatechin being the most prevalent. To assess the antioxidant activity of the methanol extract of barley, a DPPH free radical scavenging assay was conducted. The IC50 value, representing the quantity of the sample required to scavenge 50% of the DPPH free radicals, was determined to be 600.8 ± 50.50 mg TE per 100 g.

Table 3. Mean ± SD (n = 3) characteristics of barley sprouts (**BS**) powder.

Compounds[1]	Concentration in BS
TPC	302.8±10.9
Protocatechuic acid	5.8±0.2
Vanillic acid	83.8±3.2
Catechin	61.3±2.7
Epicatechin	47.8±2.0
Chlorogenic acid	20.3±0.8
Gallic acid	43.2±1.1
Ferulic acid	14.5±0.4
Sinapic acid	19.5±0.8
Myricetin	2.6±0.1
Quercetin	0.7±0.03
Kaempferol	3.3±0.08
IC_{50}^{DPPH}	600.8±50.5

Abbreviations: TPC, total phenolic content; GAE, gallic acid equivalents; IC_{50}^{DPPH}, half-maximal inhibitory concentration; TE, Trolox equivalent.

[1]Results are presented in mg 100/g, where TPC in mg GAE 100/g, IC_{50}^{DPPH} in mg TE 100/g.

Semen Quality

Spermatozoa derived from birds fed with BS + DA exhibited notably enhanced membrane functionality in comparison to the other groups (Figures 1 and 6). The interaction between time and treatment significantly influenced the functionality of the plasma membrane. As depicted in Figure 1, apart from the DA and control groups, all other groups displayed a progressive increase in sperm membrane functionality, which eventually reached a plateau towards the end of the experiment.

The integrity of the sperm plasma membrane was greatly improved in the birds treated with BS, DA, and BS + DA compared to the control group (85.29, 86.12, and 86.37 vs. 78.41, respectively) (Figure 2). Furthermore, the interaction between time and treatment had a

Table 4. Details of primer sequences used for quantitative real-time PCR in rooster's testis tissue.

Gene	Sequence (50−30)	Product length (bp)	Accession no.
StAR	Forward: TTCAGCGAGATGGAGATGTCC Reverse: GGAACACCTTACCCACGTCC	160	NM_204686
P450scc	Forward: GTTGGGTGTCTACGAGAGCG Reverse: TTGCGGTAGTCACGGTATGC	126	NM_001001756
β-actin	Forward: AGCCAACAGAGAGAAGATGACAC Reverse: CATCACCAGAGTCCATCACAATA	134	L08165.1

Abbreviations: *StAR*, steroidogenic acute regulatory protein; *P450scc*, cholesterol side-chain cleavage enzyme.

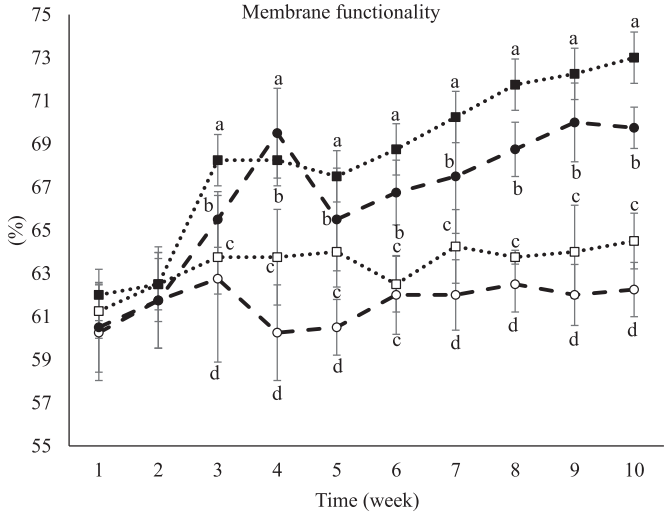

Figure 1. Weekly variation in sperm membrane functionality (The percentage of sperm with a swollen bubble around the curled flagellum in a hypo-osmotic solution) on roosters (50 wk of age; n = 8 per group) given dietary treatments for 12 successive weeks. Control (—○—, a basal diet); barley sprout (—●—, BS, 2% of basal diet); D-aspartic acid (••□••, DA, 200 mg/kg/BW) and BS + DA (••■••). [a-d]Within a week show, groups without a common superscript differed ($P <$ 0.05). Error bar = standard error of the mean.

significant impact on the integrity of the sperm plasma membrane. The groups treated with BS, DA, and BS + DA experienced a sharp increase in plasma membrane integrity during the experiment. In contrast, the control group exhibited a relatively stable trend throughout the trial.

The BS + DA group exhibited a significantly higher sperm concentration ($P < 0.01$) compared to the control, BS, and DA groups (3.69 vs. 3.09, 3.52, and 3.57). The interaction between time and treatment also had a significant impact on sperm concentration. Throughout the experiment, the BS, DA, and BS + DA groups experienced a sharp increase in sperm concentration. In

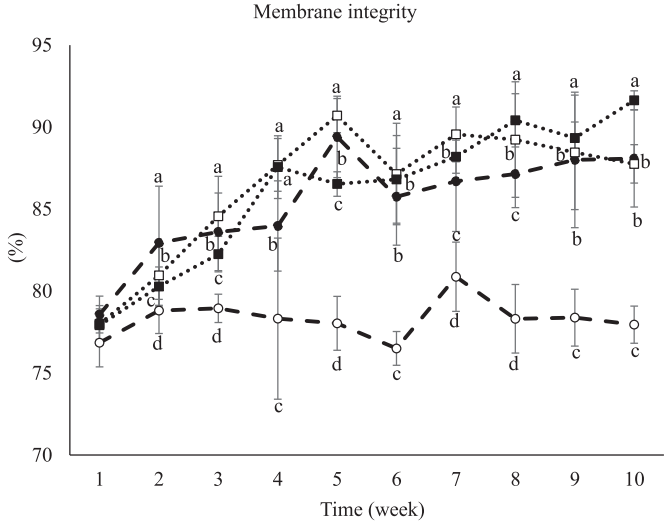

Figure 2. Weekly variation in sperm membrane integrity on roosters (50 wk of age; n = 8 per group) given dietary treatments for 12 successive weeks. Control (—○—, a basal diet); barley sprout (—●—, BS, 2% of basal diet); D-aspartic acid (••□••, DA, 200 mg/kg/BW) and BS + DA (••■••). [a-d]Within a week show, groups without a common superscript differed ($P < 0.05$). Error bar = standard error of the mean.

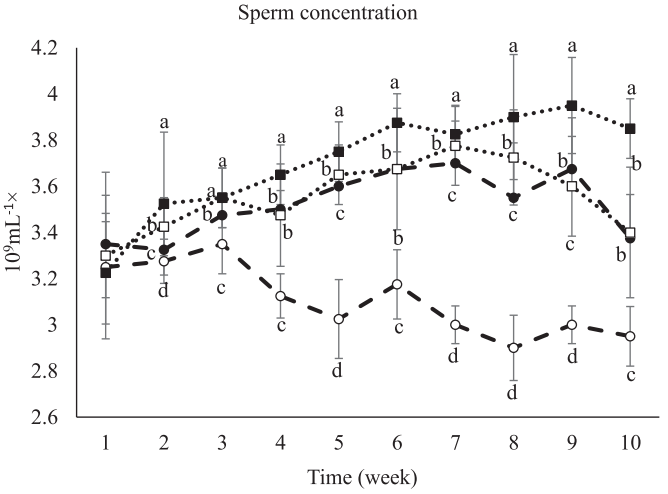

Figure 3. Weekly variation in sperm concentration on roosters (50 wk of age; n = 8 per group) given dietary treatments for 12 successive weeks. Control (—○—, a basal diet); barley sprout (—●—, BS, 2% of basal diet); D-aspartic acid (••□••, DA, 200 mg/kg/BW) and BS + DA (••■••). [a-d]Within a week show, groups without a common superscript differed ($P <$ 0.05). |Error bar = standard error of the mean.

contrast, the control group showed a relatively stable trend over the course of the trial (Figure 3).

Both the BS and BS + DA groups exhibited significantly higher total and forward motility in comparison to the control and DA groups. Notably, the birds that received BS + DA treatment displayed an approximate 8% increase in both total and forward motilities when compared to the control birds (88.82 and 78.90 vs. 81.33 and 70.85, respectively). In addition to the treatment effect, there was also a significant interaction between time and treatment for both sperm total and forward motilities. Figure 4 illustrates the interaction between time and treatment for sperm total motility, revealing an increasing trend for the BS and BS + DA groups towards the end of the experiment. Conversely, the control and DA groups displayed a relatively steady trend in the percentage of sperm total motility during the 1st wk (80.75, 82, 82.5, and 82 for Control, BS, DA, and BS + DA, respectively), which then reached 80.75, 91.25, 82, and 92.75 by the 10th wk.

Figure 5 demonstrates the combined influence of time and treatment on the forward motility of sperm. The control group of birds exhibited a relatively consistent pattern over time. Conversely, both the BS and BS + DA groups displayed a noticeable upward trend throughout the duration of the experiment. Notably, the administration of BS + DA led to significant enhancements in forward motility (approximately 13%) during the final week of sperm evaluation, when compared to the control group's forward motility of 70.85%.

Lipid Peroxidation

MDA levels exhibited a significant reduction of more than 20% across all groups in comparison to the control group (Figure 7), suggesting antioxidant property of BS and DA.

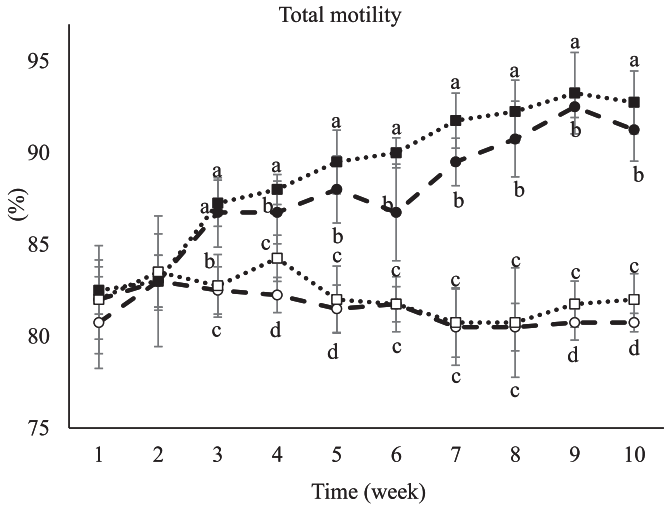

Figure 4. Weekly variation in sperm total motility on roosters (50 wk of age; n = 8 per group) given dietary treatments for 12 successive weeks. Control (—○—, a basal diet); barley sprout (—●—, BS, 2% of basal diet); D-aspartic acid (••□••, DA, 200 mg/kg/BW) and BS + DA (••■••). [a-d]Within a week show, groups without a common superscript differed (P< 0.05). Error bar = standard error of the mean.

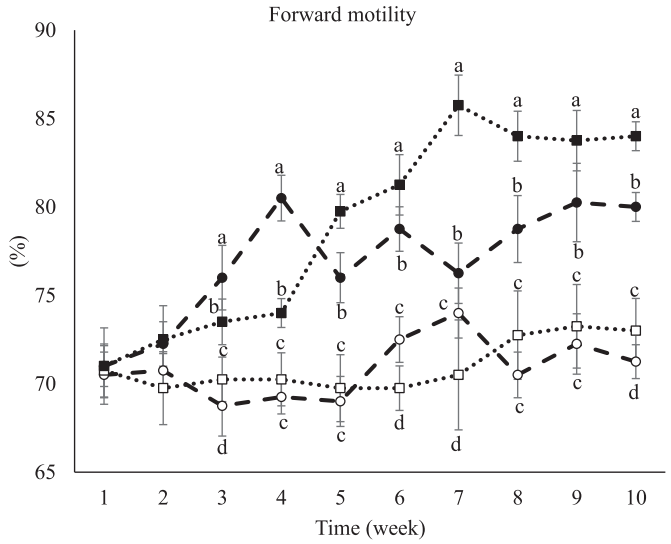

Figure 5. Weekly variation in sperm forward motility on roosters (50 wk of age; n = 8 per group) given dietary treatments for 12 successive weeks. Control (—○—, a basal diet); barley sprout (—●—, BS, 2% of basal diet); D-aspartic acid (••□••, DA, 200 mg/kg/BW) and BS + DA (••■••). [a-d]Within a week show, groups without a common superscript differed (P < 0.05). Error bar = standard error of the mean.

Serum Testosterone, Sperm Penetration, Fertility Rate, and Hatchability

The combination of dietary BS + DA supplementation resulted in the highest serum concentrations of testosterone, as well as the highest sperm penetration, fertility rate, and hatchability percentage (Table 5). Notably, testicular weights were significantly increased with the addition of BS + DA supplementation (P < 0.01). Correlation analyses revealed a strong positive correlation between total motility and fertility (+0.89, P < 0.01), while a negative correlation was observed between total motility and body weight (-0.03, P > 0.05). Additionally, a positive correlation was found between hatchability and testes weight, as well as testosterone. However, a significant negative correlation of -0.03 (P > 0.05) was observed when comparing hatchability to body weight (Table 6).

mRNA Expression

The findings presented in Figure 8 illustrate the impact of treatments on the mRNA expressions of P450scc and StAR in the testicles. It was observed that the combination of BS + DA significantly increased the relative mRNA expression levels of P450scc and StAR (P < 0.01).

DISCUSSION

The objective of this research was to investigate the potential of incorporating BS and DA into the diet as a means to alleviate age-related sub-fertility in aging breeder roosters. Numerous studies have highlighted the reproductive advantages of cereal sprouts, attributing them to the presence of flavonoids and phenolic compounds that are synthesized during the soaking and sprouting process (Ge et al., 2021). Medicinal plants and their potential to improve fertility have been recently investigated in humans and animals. In this trial, sprouted barley exhibited a rich presence of phenolic compounds, with vanillic acid, catechin, epicatechin, Myricetin, quercetin and kaempferol (Similar mechanism of action polyphenols (being the most prevalent, Typically, these bioactive compounds are commonly referred to as antioxidants because of their ability to scavenge reactive oxygen species. It can prevent the peroxidation of lipids. Free radicals attack fatty acids, steal their electrons, and convert them to free radicals. Since the formed fatty acid radicals are unstable, they react with oxygen and produce peroxyl-fatty acid radicals. Peroxyl- fatty acid radicals accelerate this chain by acting as free radicals and more peroxyl-fatty acid radicals. Quercetin is a strong antioxidant that eliminates oxygen radicals and prevents peroxyl fatty acid formation (Wright et al., 2014; Christina et al., 2015)

In this investigation, the TPC value of the methanol extract of sprouted barley corresponded to the findings of 367.7 GAE/100 g (Tumbas Šaponjac et al., 2016) and 381.7 ± 7.4 mg of GAE/100 g (Tumbas Šaponjac et al., 2019). The IC_{50} value, which represents the quantity of the sample needed to scavenge 50% of a specific concentration of DPPH free radicals, was determined to be 600.8 ± 50.5 mg TE/100 g DW, which aligns with the value of 666 ± 62.6 mg TE/100 g DW reported by Alvarez-Jubete et al. (2010). However, it is notably lower than the value of 786.7 ± 9.3 TE/100 g DW reported by Tumbas Šaponjac et al. (2019) and higher than the value of 559.7 TE/100 g DW reported by

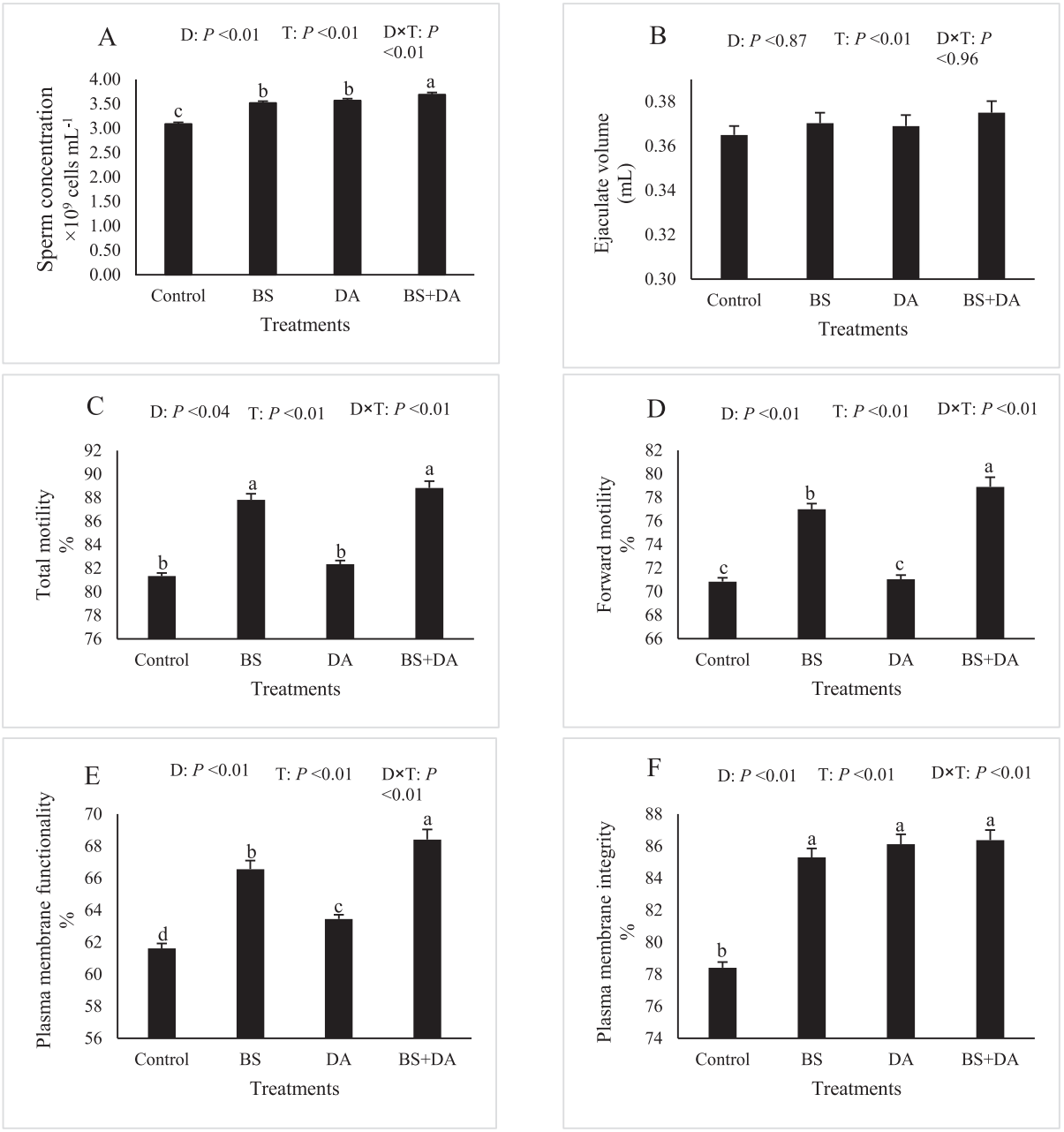

Figure 6. Means of sperm concentration (A), ejaculate volume (B), total motility (C), forward motility (D), plasma membrane functionality (E), plasma membrane integrity (F) in aging broiler breeder roosters (50 wk of age; n = 8 per group) fed by experimental treatments (Control, a basal diet; BS, 2% of basal diet; DA, 200 mg/kg/BW and BS + DA) for 12 successive weeks. Abbreviations: BS, barley sprout; DA, D-aspartic acid; D, diet (BS × DA); T, time (week). [a-d]Means without a common letter differed ($P < 0.05$). Error bar = standard error of the mean.

Tumbas Šaponjac et al. (2016). These variations can be attributed to differences in genetic backgrounds, germination duration, environmental factors, agronomic practices, and the polarity of solvents employed during the extraction process.

The inclusion of dietary supplements BS, DA, or a combination of both resulted in significant reductions in body weight, and increased total and forward sperm motility (excluding DA). Additionally, these supplements improved the integrity and functionality of the plasma membrane. These enhancements could be attributed to the antioxidant and protective properties of BS and DA. Additionally, the capabilities of BS and DA was confirmed by the reduced levels MDA in the seminal plasma of roosters fed BS and DA. Avian sperm plasma membranes are vulnerable to lipid peroxidation due to a significant presence of polyunsaturated fatty acids and a decline in antioxidant capacity with age. Therefore, enrichment of antioxidant system benefits sperm motility and plasma membrane integrity. Furthermore, there have been reports indicating that the enhancement of plasma membrane integrity and functionality could potentially be facilitated by the NMDA-R (N-methyl D-aspartic acid receptor). This receptor plays a crucial role in permitting the influx of extracellular Ca^{2+} into the cell. It is worth noting that calcium and HCO_3 anion stimulate flagellum strokes through a pathway that is dependent on adenylyl cyclase and protein kinase A

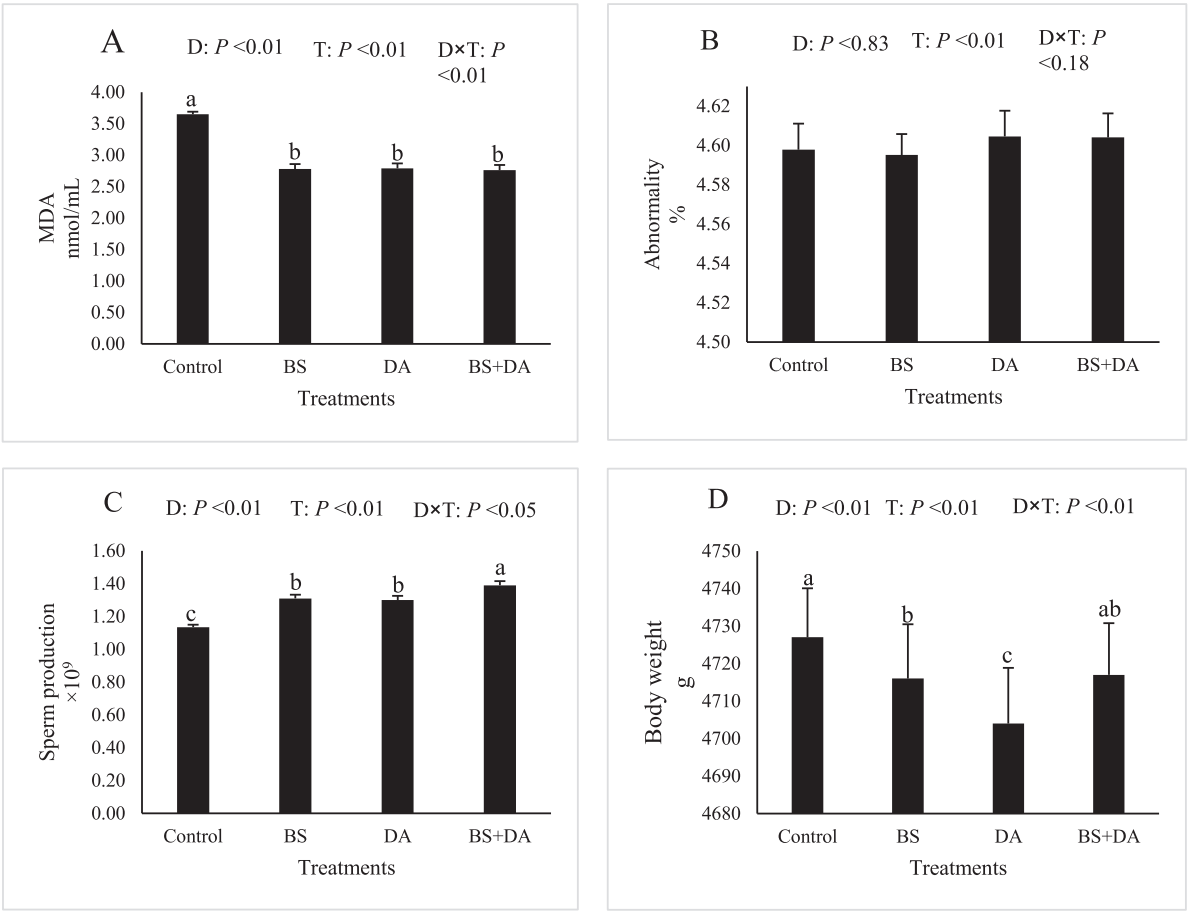

Figure 7. Means of sperm MDA (A), abnormality (B), sperm production (C), and body weight (D) in aging broiler breeder roosters (50 wk of age; n = 8 per group) fed by experimental treatments (Control, a basal diet; BS, 2% of basal diet; DA, 200 mg/kg/BW and BS + DA) for 12 successive weeks. Abbreviations: BS, barley sprout; DA, D-aspartic acid; D, diet (BS × DA); T, time (week); MDA, seminal malondialdehyde. [a-c]Means without a common letter differed ($P < 0.05$). Error bar = standard error of the mean.

(Carlson et al., 2003; Gualtieri et al., 2005). In line with the sperm abnormality, neither BS, DA, nor their combination had any effect on sperm abnormality or ejaculate volume in aging roosters. These findings differ from those reported by Ansari et al. (2018), who observed higher sperm motility in aging roosters that were fed a diet supplemented with 100 to 300 mg/kg/d of DA.

In the current study, the inclusion of BS, DA, or a combination of both in the diet had a significant impact on sperm concentration and seminal plasma concentration of MDA. Similar findings were reported by Ansari et al. (2018), who conducted a study on aging roosters and supplemented their diet with varying concentrations of DA (100−300 mg/kg/d). MDA is a byproduct of peroxidation of polyunsaturated fatty acids in cells and its levels increase due to the production of free radicals. In our study, the decrease in seminal plasma levels of MDA can be attributed to the overexpression of

Table 5. Mean (±SEM, n = 8) serum concentrations of testosterone, testes weight and reproductive end points in aging broiler breeder roosters fed by experimental treatments.

	Group				
Traits	Control	BS	DA	BS+DA	*P*-value
Testosterone (ng/mL)	$3.90^{b} \pm 0.06$	$4.07^{ab} \pm 0.04$	$4.25^{ab} \pm 0.06$	$4.35^{a} \pm 0.06$	0.04
(g) Testes weight[1]	$39.25^{c} \pm 1.43$	$40.25^{c} \pm 1.65$	$43.25^{ab} \pm 0.85$	$45.50^{a} \pm 0.28$	0.01
SP (holes mm^{-2} GD)	$81.25^{b} \pm 0.85$	$132.25^{a} \pm 1.54$	$82.75^{b} \pm 1.97$	$128.75^{a} \pm 1.75$	0.01
(%) Fertility rate[2]	75.39^{b}	84.65^{a}	76.41^{b}	85.14^{a}	0.01
(%)Hatchability[3]	73.61^{b}	83.75^{a}	83.22^{a}	82.75^{a}	0.01

Abbreviations: BS, barley sprout powder; DA, D-aspartic acid;
SP, sperm penetration (holes mm^{-2} GD, Germinal disc); SEM, standard error of the mean.
[1]Testes weight (g), left testis weight + right testis weight.
[2]Fertility rate (%), (fertile eggs number/set eggs number) × 100.
[3]Hatchability (%), (hatched eggs number/fertile eggs number) × 100.
[a-c]Within rows, different superscript letters indicate significant differences ($P < 0.05$).

Table 6. The correlation coefficient of hatchability and sperm motility vs. some reproduction traits (n = 32).

Comparison	Coefficient	*P*-value
Hatchability vs. body weight	−0.03	0.89
Hatchability vs. testes weight	+0.26	0.31
Hatchability vs. testosterone	+0.41	0.10
Total motility vs. body weight	−0.03	0.89
Total motility vs. testes weight	+0.36	0.15
Total motility vs. testosterone	+0.30	0.25
Total motility vs. fertility	+0.89	0.01
Total motility vs. hatchability	+0.48	0.05

antioxidant enzymes, such as superoxide dismutase and glutathione peroxidase, which play crucial roles in protecting biological systems against free radical damage (Ighodaro and Akinloye, 2018). Previous research has also reported the inhibitory effects of herbal antioxidants on lipid peroxidation (Teymourizadeh et al., 2020). Furthermore, there was a negative correlation between sperm motility and sperm MDA concentrations, as reported by Suleiman et al. (1996). Similarly, Slamenova et al. (2011) found that herbal antioxidants reduced epididymal sperm injury by inhibiting lipid peroxidation, thereby improving sperm motility. Consistent with these findings, the antioxidant effects of BS have been documented in male rats (Khattab et al., 2022).

The aging rooster may experience a physiological issue involving a weakened connection between the hypothalamus and the hypothalamus-hypothesis axis, potentially leading to a suppression of reproductive hormone secretion (Weil et al., 1999). The administration of DA and BS showed a stimulating effect starting from the second week, which can be attributed to their impact on testosterone production and the functioning of the testicular antioxidant system. In this study, roosters given BS + DA exhibited the highest serum concentrations of testosterone. In birds, the release of luteinizing hormone (**LH**) and follicle-stimulating hormone (**FSH**) from the adenohypophysis is regulated by the release of gonadotropin-releasing hormone (**GnRH**) from the hypothalamus. The production of steroids by leydig cells is strictly controlled by LH. The present study observed an up-regulation of the *StAR* gene in birds fed with DA or BS + DA. This gene plays a role in the production of steroid hormones by facilitating the transfer of cholesterol from the cytoplasm into the mitochondria of leydig cells. Within the mitochondria, cholesterol is converted to pregnenolone by *P450scc*, which was up-regulated by the experimental treatments. Pregnenolone serves as the precursor for the synthesis of all steroid hormones (Heng et al., 2017). Reproductive hormones, particularly testosterone and dihydrotestosterone, play a vital role in the elimination of abnormality sperm. A direct correlation between elevated testosterone levels and the proportion of viable sperm has been established (Tang et al., 2012).

Previous research has documented a decline in the mRNA expression of crucial steroidogenic enzymes, including *17b-HSD, 3b-HSD*, and *P450scc*, with advancing age (Luo et al., 1996, 2001). In our study, the mRNA levels of 2 pivotal enzymes involved in steroidogenesis, namely *StAR* and *P450scc*, were found to be augmented by DA. This enhancement is likely attributed to the upregulation of *LHR* transcripts and/or activation of the adenylyl cyclase/cAMP/protein kinase A and mitogen-activated protein kinase (***MAPK***) pathways (Di Fiore et al., 2016). The observed effects of DA on *StAR* and *P450scc* expression in this study align with the findings of Ansari et al. (2018), who administered varying concentrations of DA to aging roosters

Fertility assessment plays a crucial role in validating the outcomes of *in vitro* experiments (Shahverdi et al., 2015; Fattah et al., 2017b). Our findings revealed that both BS and BS + DA led to increases in fertility rate and sperm penetration that is confirming our previously published report (Yarmohammadi Barbarestani et al., 2023). Interestingly, Ansari et al. (2018) reported a positive influence of DA on fertility rate, which contrasts with our findings. Previous research has highlighted a

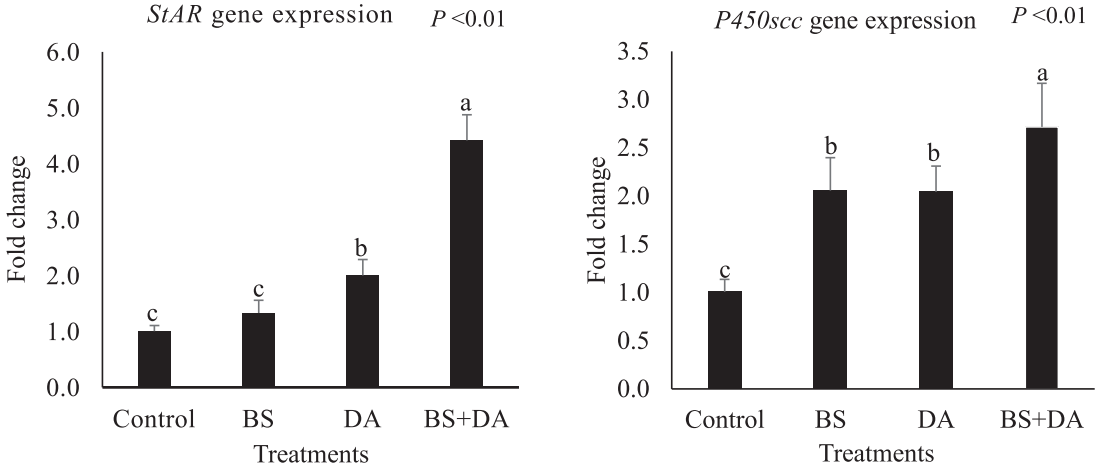

Figure 8. Relative mRNA expression of *StAR* (left) and *P450scc* (right) and levels in aging broiler breeder roosters (50 wk of age; n = 8 per group)' testes fed by experimental treatments (Control, a basal diet; BS, 2% of basal diet; DA, 200 mg/kg/BW and BS + DA) for 12 successive weeks. Abbreviations: *P450scc*, cholesterol side-chain cleavage enzyme; *StAR*, steroidogenic acute regulatory protein; BS, barley sprout; DA, D-aspartic acid. [a-c]Means without a common letter differed ($P < 0.05$). Error bar = standard error of the mean.

strong connection between the health of the sperm membrane and its ability to penetrate the oocyte (Malo et al., 2010). Furthermore, our results demonstrated that BS, DA, and their combination improved hatchability, consistent with the findings of Ansari et al. (2018). Although the precise mechanism of sperm transportation within the reproductive tract of hens remains unclear, enhanced plasma membrane integrity and functionality, along with sperm motility, facilitate sperm uptake into sperm storage tubules and enhance fertility (Sasanami et al., 2013). In this context, we observed a significant positive correlation between total motility and fertility (+0.89). Additionally, we found negative correlations between hatchability and body weight (-0.03), as well as between total motility and body weight (-0.03). Generally, excessive body weight (aging) in roosters leads to a decline in reproductive performance.

CONCLUSIONS

In this investigation, the addition of BS, DA, or a combination of both to the diet exhibited advantageous effects on the reproductive capabilities of aging roosters. These effects were observed through the enhancement of sperm quality over time, prevention of lipid peroxidation, up-regulation of steroidogenic genes, and improvements in fertility and hatchability. Nevertheless, additional research is required to fully elucidate the reproductive benefits of BS in aging broiler breeder roosters.

ACKNOWLEDGMENTS

The authors gratefully thank Gorgan University of Agricultural Sciences and Natural Resources (Gorgan, Iran) and MAMco broiler integration (Babol, Iran) for facility support.

DISCLOSURES

The authors declare no conflicts of interest.

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