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Fermented bamboo powder enhances testicular function and reproductive performance by modulating spermatogenesis, hormonal profiles, and autophagy in yellow-feathered roosters

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ABSTRACT

Objective: To investigate the effects of Fermented Bamboo Powder (FBP) supplementation on testicular development and reproductive performance in yellow-feathered roosters.

Methods: A total of 300 roosters were randomly assigned to two groups ($n=150$ per group) and subjected to a 77-day feeding trial. The control group received a standard basal diet, while the experimental group was fed the same basal diet supplemented with FBP in a stepwise dosing regimen: 1.0 g/kg during Phase I (days 1–22), 2.0 g/kg during Phase II (days 23–45), 4.0 g/kg during Phase III (days 46–60), and 6.0 g/kg during Phase IV (days 61–77). Testicular morphology and spermatogenesis were evaluated using hematoxylin and eosin staining and gonadosomatic index. Serum lipids and reproductive hormones were measured with assay kits and enzyme linked immunosorbent assay. Autophagy-related proteins were analyzed *via* Western blot, and spermatogenesis-related genes were quantified by quantitative real-time polymerase chain reaction.

Results: FBP supplementation improved left testis width, length, and thickness, as well as right testis width ($P<0.05$). Both individual and total testis weights were markedly higher in the FBP group. Histological analysis revealed notable improvements in testicular architecture, including increased seminiferous tubule diameter, epithelial height, luminal area, and Johnsen's score. At the molecular level, FBP upregulated mRNA expression of spermatogenesis-related genes such as *CREM*, *PCK2*, *DDX4*, *GDNF*, *SPAG6*, *SPAG16*, and *SPAG17* ($P<0.05$). There was a significant increase in total protein and a reduction in triglyceride concentrations. Hormonal evaluations indicated significantly elevated serum testosterone and FSH levels in the FBP group. Importantly, FBP enhanced the expression of autophagy-related proteins beclin-1 and ATG5, suggesting increased autophagic activity. Additionally, phosphorylated mTOR levels were significantly increased.

Conclusions: FBP supplementation positively affects testicular

morphometry, endocrine profiles, and molecular pathways involved in spermatogenesis and autophagy, supporting its potential role in improving male reproductive performance in poultry.

KEYWORDS: Yellow-feather rooster; Fermented bamboo powder; Spermatogenesis; Testicular histology; Reproductive Performance

Key Points

Question: Can Fermented Bamboo Powder (FBP) supplementation enhance testicular growth, spermatogenesis, and reproductive hormone levels in yellow-feathered roosters?

Results: FBP supplementation enhanced testis size, weight, and histology, upregulated spermatogenesis-related genes, improved serum testosterone and FSH, modulated protein and triglyceride levels, and increased autophagy-related proteins and phosphorylated mTOR.

Meaning: FBP supplementation boosts testicular development, hormones, and molecular pathways, supporting improved male reproductive performance in poultry.

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1. Introduction

The poultry industry plays a vital role in ensuring global food security, and improving the reproductive efficiency of breeder flocks that is key to increasing productivity and supporting sustainable development[1]. A primary aim of breeder operations is to produce the maximum number of fertile eggs, which directly results in more healthy, one-day-old chicks[2]. Roosters are essential to this process, particularly because breeder flocks typically maintain a low male-to-female ratio[3]. However, male infertility remains a major concern, contributing to about 30% of fertility problems in poultry production[4]. Several factors can affect the reproductive performance of roosters, including age, nutrition, climate, and management practices[5]. Among these, age is one of the most critical; after 45 weeks, many roosters have a decline in sperm production and testosterone levels, which can lower fertility[6,7]. This presents a challenge for maintaining reproductive performance in older breeding stock. Specific dietary supplements, such as functional feed additives, can improve fertility in aging roosters[3,6,8,9], enhancing semen quality and overall reproductive health, and offering a practical solution to fertility decline in commercial poultry operations.

Bamboo powder, derived from the bamboo plant, is increasingly recognized as a valuable natural supplement across various fields, including agriculture and animal husbandry. Bamboo is widely distributed in tropical and subtropical regions, particularly throughout Asia, Africa, and South America[10]. Valued for its rapid growth and sustainability, bamboo has a long history of traditional use in construction, medicine, and as a food source in many cultures[10]. Bamboo powder is rich in essential nutrients such as amino acids, minerals, and dietary fiber, and contains a variety of bioactive compounds such as phenols, flavonoids, and lignans, which contribute to its antioxidant, anti-inflammatory, and antibacterial properties[11–13]. Moreover, bamboo powder exhibits immunomodulatory effects, and helps improve overall health and resilience[14]. In poultry, these bioactive components are believed to support animal health by enhancing digestive function, strengthening immune responses, and mitigating oxidative stress[15]. Importantly, recent research has highlighted the potential of bamboo powder to optimize reproductive health[16]. Natural dietary supplements are of particular interest because they can improve semen quality, stimulate spermatogenesis, support testicular morphology, and enhance male fertility[17,18].

Despite its promising attributes, the full reproductive potential of bamboo powder, particularly in its fermented form, remains underexplored. Fermentation, especially when mediated by microbial enzymes, has been shown to enhance the bioactivity, digestibility, and absorption of plant-based supplements[19]. This process often generates additional bioavailable compounds with antioxidant,

antimicrobial, and anti-inflammatory properties that are either absent or less potent in unfermented counterparts[20]. Fermented Bamboo Powder (FBP), therefore, contributes to improved systemic health, as evidenced by its beneficial effects on serum biochemical parameters, immune function enhancement, and modulation of fecal microbial composition in growing-finishing pigs[21]. Previous research on fermented plant products such as fermented soybean and tea leaves has demonstrated improvements in reproductive performance and cellular regeneration in livestock[22,23].

While the use of bamboo powder as a dietary supplement for livestock remains an emerging area of research, its potential to enhance reproductive performance, particularly in poultry, warrants further investigation. To date, no studies have specifically examined the effects of FBP on spermatogenesis or overall male reproductive performance in roosters. Therefore, this study was designed to evaluate the impact of FBP supplementation on spermatogenesis, testicular histomorphology, reproductive hormone concentrations, and blood biochemical parameters in yellow-feathered roosters. In addition, the study aimed to assess the expression of key autophagy-related proteins in testicular tissue to elucidate the underlying mechanisms through which FBP may support testicular function and germ cell development.

2. Methods

2.1. Experimental design

A total of 300 healthy, one-day-old male yellow-feathered rooster chicks were used in the study. The chicks were randomly divided into two dietary groups, with 150 chicks in each group. Each group was further divided into five replicates, and each replicate contained 30 chicks. The control group received a standard basal diet, while the experimental group was fed the same basal diet supplemented with FBP in a stepwise dosing regimen: 1.0 g/kg during Phase I (days 1–22), 2.0 g/kg during Phase II (days 23–45), 4.0 g/kg during Phase III (days 46–60), and 6.0 g/kg during Phase IV (days 61–77). This graded supplementation strategy was designed to align with the yellow-feathered roosters' natural growth and reproductive maturation, facilitating physiological adaptation and minimizing dietary stress. Although FBP was administered at different concentrations across the four phases, the experimental group was treated as a single group for statistical analysis. The overall effects of FBP supplementation were evaluated by comparing the cumulative outcomes of the experimental group against the control group over the entire 77-day period. At the end of Phase IV, samples including blood, testicular tissues, and reproductive organs were collected from ten roosters per group for analysis of spermatogenesis, testicular histology, hormone levels, blood biochemistry, and autophagy-

related protein expression. Additionally, the chicks' body weights were recorded at the beginning and at the end of the experiment. All yellow-feathered roosters were housed under controlled temperature and lighting conditions, with *ad libitum* access to feed and water.

2.2. Diet composition and source

The basal diet used in this study was sourced from Jiangsu Yancheng Xiling Agricultural Science and Technology Co., Ltd., Jiangsu, China, and its composition is detailed in Supplementary Table 1. The diet was formulated to meet the nutritional requirements of adult poultry according to National Research Council (NRC) 1994 Nutrient Requirements of Poultry, ensuring adequate energy, protein, vitamins, and minerals to support normal growth, maintenance, and reproductive function. The FBP was supplied by Zhejiang Muyi Xiangzhu Biotechnology Co., Ltd., China, utilizing proprietary fermentation technology developed by the Research Institute of Global 3E, Kyoto, Japan. The primary nutritional components of FBP include crude protein, crude fiber, crude fat, crude ash, acid-soluble protein, and acid-washing lignin, as listed in Supplementary Table 2. All yellow-feathered roosters had *ad libitum* access to feed and water throughout the study, and FBP supplementation was added to the basal diet as described in the experimental design.

2.3. Sample collection

At the end of the experimental period, the yellow-feathered roosters were euthanized humanely by cervical dislocation, following standard ethical guidelines for poultry research. Blood samples were immediately collected from the wing vein of ten roosters using sterile syringes. The collected blood was allowed to clot at room temperature for 30 min, then centrifuged at 1 500×g for 10 min at 4 °C to separate the serum, which was stored at −20 °C until further analysis. Both testes were carefully excised. One testis was rinsed in phosphate-buffered saline (PBS, pH 7.4) and stored at −80 °C for subsequent molecular analyses, while the other testis was fixed in Bouin's solution for histological examination. All procedures were conducted promptly to minimize post-mortem changes and preserve sample integrity.

2.4. Testicular morphology and spermatogenetic analysis

For histological analyses, testes from all ten roosters per group were collected and weighed to calculate the gonadosomatic index (GSI), defined as the ratio of testis weight to body weight[20]. Following collection, testes were fixed overnight in Bouin's solution and subjected to a graded dehydration process using ethanol solutions, followed by xylene, before embedding in paraffin. Tissue sections of 5 µm thickness were obtained using an RM2235 microtome (Leica Biosystems Inc., Germany), deparaffinized in xylene, and rehydrated

through a series of ethanol solutions. Sections were then stained with hematoxylin and eosin (H & E) for histological examination. Images of the testes were captured and analyzed under a Nikon ECLIPSE E200 microscope (Nikon Instruments Inc., U.S.A.).

To assess seminiferous tubule structure, measurements of tubule diameter, epithelial height, tubule area, lumen diameter, and lumen area were taken at five randomly selected sites within each testis using Image-Pro Plus software version 6.0. Spermatogenesis was evaluated by scoring the morphology and progression of the germinal epithelium in seminiferous tubules based on established criteria[26–28], with each tubule cross-section assigned a grade from 1 to 5, as detailed in Supplementary Table 3. Histological assessments were performed in a blinded manner, where the examiner was unaware of the group assignments, to reduce observer bias and improve the reliability of the measurements.

2.5. Lipid parameter analysis in serum

At the end of the experiment, blood samples were collected from the wing vein of ten randomly selected yellow-feathered roosters per group. The serum was separated by centrifugation at 1 500×g for 10 min at 4 °C and then stored at −20 °C until further analysis. Serum samples were analyzed for total cholesterol, triglycerides, low-density lipoprotein (LDL), total protein, and uric acid using commercial assay kits (Nanjing Jiancheng Bioengineering Institute, China), following the manufacturer's instructions.

The sensitivity for cholesterol, triglyceride, LDL, total protein, and uric acid assays ranged from 0.05 to 0.10 mg/dL. Intra-assay and inter-assay coefficients of variation (CVs) for all parameters were below 6% and 9%, respectively. No significant cross-reactivity was reported by the manufacturer. All measurements were performed in duplicate.

2.6. Assessment of reproductive hormone levels

At the end of the experimental period, blood samples were collected from the wing vein of ten roosters per group into EDTA tubes. Plasma was separated by centrifugation at 1 500×g for 10 min and stored at −20 °C until hormone analysis.

Plasma concentrations of total testosterone (nmol/L), luteinizing hormone (LH, mIU/mL), and follicle-stimulating hormone (FSH, IU/L) were determined using validated enzyme linked immunosorbent assay (ELISA) kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The sensitivity of the assays was 0.1 nmol/L for testosterone, 0.05 mIU/mL for LH, and 0.05 IU/L for FSH. Intra-assay CVs were <6%, and inter-assay CVs were <9% for all hormones. The assays showed no notable cross-reactivity with related hormones. All samples were analyzed in duplicate to ensure accuracy.

2.7. Western blotting

Total protein was extracted from frozen chicken testicular tissue using radio immunoprecipitation assay (RIPA) lysis buffer supplemented with 1 mmol/L phenyl methane sulfonyl fluoride (PMSF) (Beyotime Biotechnology, Shanghai, China) following the manufacturer's protocol. Protein concentration was quantified using the BCA Protein Assay Kit (Beyotime, Shanghai, China). Equal amounts of total protein (20–30 µg) were separated on 4%–20% sodium dodecyl sulfonate polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred to methanol-activated polyvinylidene fluoride (PVDF) membranes (Millipore, USA) using a wet transfer system at 70 V for 1 h under constant voltage. Following transfer, membranes were blocked with 5% bovine serum albumin (BSA) in TBST (Tris buffered saline containing 0.1% Tween-20) for 2 h at room temperature to reduce nonspecific binding.

Membranes were incubated overnight at 4 °C with primary antibodies (Proteintech, USA) diluted in TBST containing 5% BSA. The antibodies used included anti-Bcl-1 (1:1 000), anti-ATG5 (1:1 000), anti-phospho-mTOR (p-mTOR) (1:1 000), anti-total mTOR (1:1 000), and anti-GAPDH (1:5 000) as the internal control. After washing the membranes three times with TBST, they were incubated with the secondary antibody for 60 min at room temperature.

Protein bands were visualized using enhanced chemiluminescence (ECL) reagent (Thermo Scientific, USA) and captured using the LAS-4000 Imaging System (Fujifilm, Japan). Band intensity was quantified using ImageJ software (NIH, USA), and the expression of target proteins was normalized against glyceraldehyde-3-phosphate dehydrogenase (GAPDH). To ensure the specificity of antibody detection, peptide blocking validation was performed for the primary antibodies (anti-Bcl-1 and anti-ATG5) using their respective immunizing peptides (Proteintech). Briefly, antibodies were pre-incubated with a 10-fold molar excess of their blocking peptides

for 2 h at room temperature before membrane incubation. Peptide-blocked blots showed absence or significant reduction of target bands, confirming antibody specificity.

2.8. Total RNA extraction and mRNA quantification

Total RNA was extracted from frozen testis tissues using TRIzol reagent (Invitrogen, USA) according to the manufacturer's protocol. Approximately 100 mg of tissue was homogenized in 1 mL of TRIzol, followed by phase separation, RNA precipitation, and washing. The RNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Scientific, USA). Samples with $A_{260/280}$ ratios between 1.8–2.0 and $A_{260/230}$ above 2.0 were considered suitable for further analysis. RNA integrity was verified by 1% agarose gel electrophoresis, ensuring the presence of sharp 28S and 18S rRNA bands.

cDNA synthesis was performed using the PrimeScript™ RT reagent Kit with gDNA Eraser (TaKaRa Bio Inc., Japan). For each 20 µL reaction, 1 µg of total RNA was used along with 4 µL of 5 PrimeScript Buffer, 1 µL of PrimeScript RT Enzyme Mix I, 1 µL of Oligo (dT) primer (50 µmol/L), 1 µL of Random 6 mers (100 µmol/L), 1 µL of gDNA Eraser, and RNase-free water to volume. The protocol included genomic DNA elimination at 42 °C for 2 min, reverse transcription at 37 °C for 15 min, and enzyme inactivation at 85 °C for 5 min. Non-template controls (NTCs) were included during cDNA synthesis to monitor for contamination.

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed using the StepOnePlus Real-Time PCR system (Applied Biosystems, USA) with SYBR® Premix Ex Taq™ II (TaKaRa, Japan). Each 20 µL reaction consisted of 10 µL SYBR Green master mix, 0.4 µmol/L each of forward and reverse primers, 2 µL of cDNA template, 0.4 µL ROX reference dye, and 6.8 µL of nuclease-free water. Primers for target genes involved in spermatogenesis

Table 1. Primers used for quantitative real-time polymerase chain reaction (qRT-PCR).

Gene	Gene name	Accession number	Sequence (5'-3')	Product length (bp)
<i>PGK2</i>	phosphoglycerate kinase 2	NM_204985.2	Forward: CCTGGAAGTCTAGAGGGTAAG Reverse: GAACTGGGGTGATACGCTGT	81
<i>CREM</i>	cAMP responsive element modulator	NM_001278132.1	Forward: AGAGAGGGAGCCCAAGAGAA Reverse: CATGGTCATCCGTCTCCAG	104
<i>DDX4</i>	DEAD-box helicase 4	NM_204708.2	Forward: CTGCTGGCATTGCTATGGA Reverse: TCTGCCAGAGTTAGCCATCC	111
<i>GDNF</i>	glial cell derived neurotrophic factor	XM_004937198.3	Forward: TTCTCCACGTCTCTCTCTCG Reverse: TCTGGCATATTAGCGGCGG	244
<i>SPAG6</i>	sperm associated antigen 6	NM_001199586.1	Forward: GAGATTATTGGAGGTATGAG Reverse: CACCAGCAGCGTAAGTAG	106
<i>SPAG16</i>	sperm associated antigen 16	XM_421865.5	Forward: AGGGCTATAAAGCAGGGTG Reverse: TGGCGAGAATAAGTAACATCAG	129
<i>SPAG17</i>	sperm associated antigen 17	XM_015294613.1	Forward: CGGCTTATCTATGAATGCT Reverse: TGAAGTCGTTCTCTCTCTA	165
<i>GAPDH</i>	glyceraldehyde-3-phosphate dehydrogenase	NM_205518.2	Forward: GCCCTCTTCCAGCCATCTTT Reverse: CAATGGAGGGTCCGGATTCA	151

(*PGK2*, *CREM*, *DDX4*, *GDNF*, *SPAG6*, *SPAG16*, and *SPAG17*), as well as the reference gene *GAPDH*, were designed based on sequences obtained from GenBank. Their specificity was confirmed by NCBI BLAST and amplicon sizes were verified using 2% agarose gel electrophoresis (Table 1).

The thermocycling protocol was initiated with an initial denaturation at 95 °C for 30 s, followed by 40 cycles of denaturation at 95 °C for 5 s and annealing/extension at 60 °C for 30 s. A melting curve analysis was performed from 60 °C to 95 °C in 0.3 °C increments every 15 s to verify amplification specificity and rule out primer-dimer formation. Each reaction was performed in triplicate, and non-template controls were included for every primer set to ensure no contamination in qRT-PCR reactions.

The relative expression of target genes was quantified using the $\Delta\Delta C_t$ method, with *GAPDH* serving as the internal control. The $2^{-\Delta\Delta C_t}$ formula was applied to calculate the fold-change in gene expression. C_t values were averaged across triplicates for each sample.

2.9. Statistical analysis

All statistical analyses were performed using SPSS software (version 25.0; IBM Corp., Armonk, NY, USA). Prior to conducting inferential tests, the data were assessed for normality using the Shapiro–Wilk test and for homogeneity of variances using Levene's test. Since both assumptions were met, an independent samples Student's *t*-test was applied to evaluate differences between treatment groups. In cases where assumptions were violated, appropriate data transformations or non-parametric alternatives (*e.g.*, Mann–Whitney *U* test) were considered; however, no transformation was ultimately required. The analysis protocol was adapted from previous studies[24,25]. All data are expressed as mean $\pm$ standard deviation (SD). A *P*-value less than 0.05 was considered statistically significant.

2.10. Ethics statement

The experimental protocol and procedures were conducted in accordance with the Guidelines for the Care and Use of Animals and were approved by the Institutional Animal Care and Ethical Committee of Nanjing Agricultural University, Nanjing, China. The study was performed under License Number SYXK (Su) 2022–0031; dated 10 July, 2023.

3. Results

3.1. Analysis of the bioactive components in the FBP pre- and post-fermentation

To determine the bioactive profile of FBP before and after fermentation,

Table 2. Bioactive components in the fermented bamboo powder pre- and post-fermentation.

Compound	Before fermentation (mg/100 g)	After fermentation (mg/100 g)
Ferulic acid	5.2	7.6
p-Coumaric acid	3.8	5.3
Caffeic acid	2.9	4.2
Protocatechuic acid	4.1	6.0
p-Hydroxybenzoic acid	3.5	5.1
Catechin	6.0	8.6
Syringic acid	2.7	4.0
Chlorogenic acid	3.2	4.5
β -Sitosterol	0.8	1.1
Campesterol	0.6	0.9
Stigmasterol	0.5	0.8
Cholesterol	0.4	0.6
Ergosterol	0.3	0.5
Stigmastanol	0.2	0.4
Phytosterols	2.4	3.6
Tannins	1.5	2.2
Saponins	1.2	1.6
Flavonoids	4.0	5.4

high-performance liquid chromatography coupled with tandem mass spectrometry (HPLC-MS/MS) was conducted by the Research Institute of Global 3E, Kyoto, Japan, with the analytical results presented in Table 2. Fermentation markedly increased the levels of bioactive compounds in the substrate. Phenolic acids, including ferulic acid, p-coumaric acid, caffeic acid, protocatechuic acid, p-hydroxybenzoic acid, catechin, syringic acid, and chlorogenic acid, were all elevated post-fermentation. Sterols such as β -sitosterol, campesterol, stigmasterol, cholesterol, ergosterol, and stigmastanol, as well as total phytosterols, also increased. Additionally, non-phenolic compounds, including tannins, saponins, and flavonoids, showed higher concentrations after fermentation, indicating an overall enhancement of the substrate's nutritional and functional properties.

3.2. Effects of FBP on testicular morphometric and gonadal indices in roosters

Morphometric analysis of the testes revealed that FBP supplementation significantly increased the width, length, and thickness of the left testis (all $P < 0.05$). In the right testis, a significant increase was observed in width ($P < 0.05$), while increases in length and thickness showed positive trends but did not reach statistical significance ($P > 0.05$). Both left and right testis weights were significantly higher in the FBP-supplemented group ($P < 0.05$) compared to the control group, resulting in a significantly greater total testis weight ($P < 0.05$). Although the GSI was higher in the FBP group, the difference was not statistically significant ($P > 0.05$). Body weight was also unaffected by FBP supplementation ($P > 0.05$) (Table 3).

Table 3. Effects of fermented bamboo powder on body weight and testicular morphometric and gonadal indices in roosters.

Parameters	Dietary treatments		P value
	Control	FBP	
Body weight, kg	1.93±0.21	2.23±0.23	0.75
Left testis width, mm	20.10±1.23	23.10±2.14*	0.04
Right testis width, mm	18.80±0.12	22.32±1.05*	0.03
Left testis length, mm	40.52±0.47	45.36±0.36*	0.04
Right testis length, mm	38.87±0.24	42.27±0.13	0.07
Left testis thickness, mm	20.21±0.21	26.03±1.41*	0.04
Right testis thickness, mm	19.72±0.43	21.01±1.52	0.09
Left testis weight, g	10.50±0.59	13.50±0.05*	0.03
Right testis weight, g	9.30±0.15	11.20±0.21*	0.04
Total testes weight, g	19.80±2.23	24.70±1.12*	0.02
Gonadosomatic index (GSI), % [†]	1.03±0.05	1.11±0.01	0.06

Data are presented as the mean±SD for each group ($n=10$ per group). Superscript symbols indicate the level of statistical significance, with * representing $P<0.05$. Means with asterisks within the same row are significantly different from each other ($P<0.05$). [†]Gonadosomatic index=(Total testes weight/Body weight)×100. FBP: fermented bamboo powder.

3.3. Effects of FBP on testicular morphology and histomorphometric evaluation in roosters

Dietary supplementation with FBP significantly enhanced testicular structure and spermatogenic activity in roosters. Histomorphometric analysis revealed notable increases in seminiferous tubule diameter ($P<0.05$), seminiferous epithelium height ($P<0.001$), seminiferous tubule area ($P<0.05$), and luminal area ($P<0.05$) in the FBP group compared to controls. However, lumen diameter did not differ significantly ($P>0.05$). Additionally, Johnsen's score, which reflects spermatogenic efficiency, was significantly higher in FBP-treated roosters ($P<0.01$), as shown in Figure 1 and Figure 2.

3.4. Effects of FBP on blood parameters in roosters

The effects of FBP supplementation on blood parameters in

yellow-feathered roosters are summarized in Table 4. No significant differences were observed in total cholesterol ($P=0.32$), uric acid ($P=0.23$), or LDL levels ($P=0.38$) between the control and FBP-treated groups. In contrast, total protein was significantly higher ($P=0.02$), and triglyceride levels were significantly lower ($P=0.04$) in the FBP group, indicating a modulatory effect of FBP on protein and lipid metabolism.

3.5. Effects of FBP on mRNA expression of spermatogenesis-related genes

To explore the impact of FBP on spermatogenesis in roosters, the mRNA expression levels of key spermatogenesis-related genes (*CREM*, *PCK2*, *DDX4*, *GDNF*, *SPAG6*, *SPAG16*, and *SPAG17*) in the testis were evaluated. The results were compelling, showing that FBP supplementation led to a significant increase in the mRNA

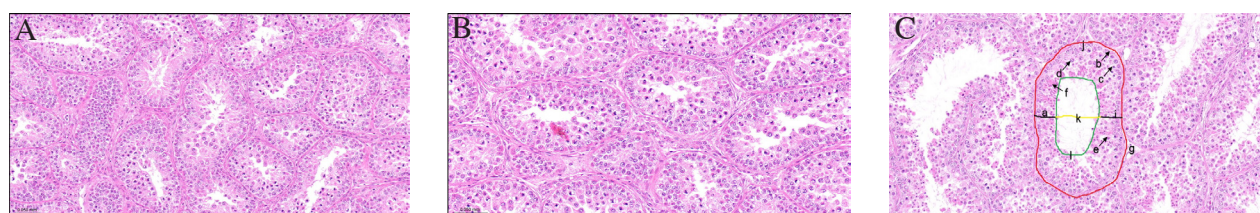


Figure 1. Histological examination of testicular tissues in the control and fermented bamboo powder-supplemented roosters. Representative hematoxylin and eosin (H & E)-stained testis sections from the control (Con) group (A) and fermented bamboo powder (FBP) group (B). Seminiferous tubule area (μm^2) of the FBP group (C). Roosters in the FBP group received a basal diet supplemented with 1 g, 2 g, 4 g and 6 g/kg FBP. Sample size: $n=10$ per group. Morphological features assessed include: (a) seminiferous tubule diameter (long black line), (b) spermatogonia, (c) primary spermatocyte, (d) secondary spermatocyte, (e) spermatid, (f) spermatozoon, (g) Leydig cell, (i) seminiferous epithelial height (short black line), (j) seminiferous tubule area (μm^2) (Red curve), (k) lumen diameter (μm) (yellow line), and (l) lumen area (μm^2) (green curve). Images were captured at 10× magnification.

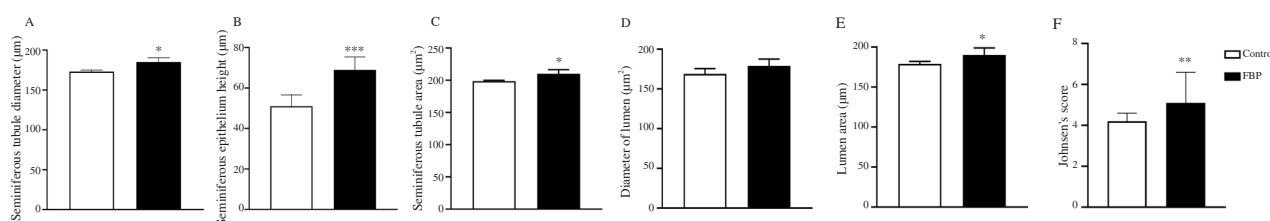


Figure 2. Histomorphometric analysis of rooster testes following dietary fermented bamboo powder supplementation. Quantitative measurements ($n=10$ per group) include: (A) seminiferous tubule diameter (μm), (B) seminiferous epithelial height (μm), (C) tubule area (μm^2), (D) lumen diameter (μm), (E) lumen area (μm^2), and (F) Johnsen's testicular score. Data are shown as mean±SD. $P<0.05$, $^{**}P<0.01$, $^{***}P<0.001$.

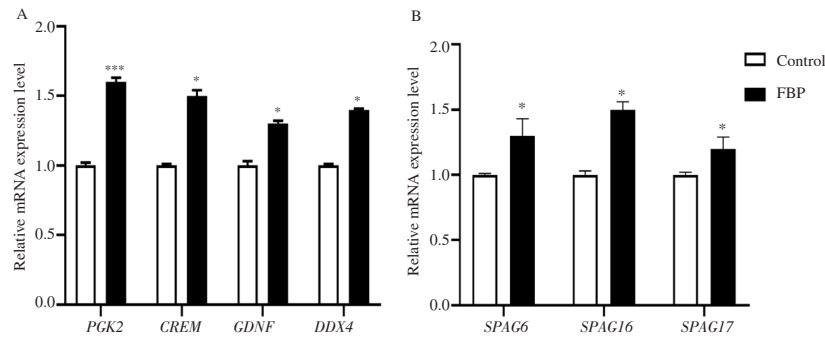


Figure 3. Effect of fermented bamboo powder on mRNA expression of spermatogenesis- and sperm motility-related genes in rooster testes. (A) Spermatogenesis-related genes; (B) Sperm motility-related genes. Data are shown as mean $\pm$ SD ($n=10$). Bar graphs represent mean expression levels, with error bars indicating SD. Significant differences between groups are denoted by asterisks: * $P<0.05$, *** $P<0.001$.

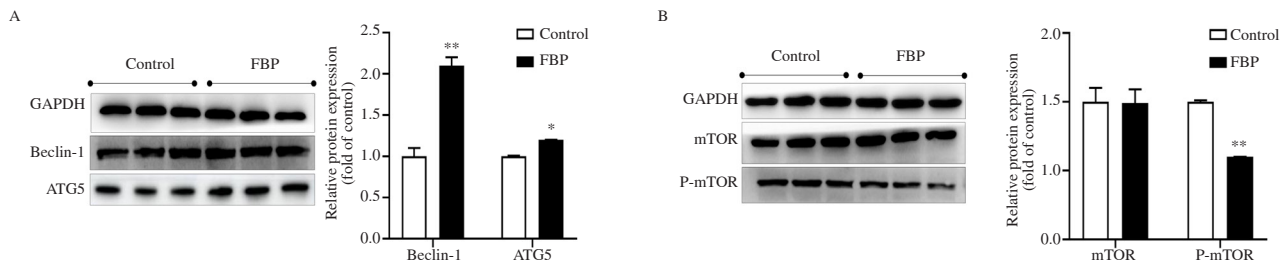


Figure 4. Effects of fermented bamboo powder on autophagy-related protein expression in rooster testes. (A) Representative Western blots of Beclin-1 and ATG5 in testicular tissue ($n=8$). Quantification of Beclin-1 and ATG5 protein levels. (B) Representative Western blots of mTOR, and phosphorylated mTOR (p-mTOR). Quantification of mTOR and p-mTOR protein levels. Data are expressed as mean $\pm$ SD. Asterisks indicate significant differences between groups: * $P<0.05$, ** $P<0.01$.

expression of *PGK2*, *CREM*, *GDNF* and *DDX4* genes compared to the control group, with statistical significance ($P<0.05$) (Figure 3A). Furthermore, as shown in Figure 3B, the expression levels of *SPAG6*, *SPAG16*, and *SPAG17* were significantly higher in the FBP supplementation group than in the control group ($P<0.05$).

3.6. Effects of FBP on plasma reproductive hormones in roosters

Dietary supplementation with FBP significantly influenced plasma reproductive hormones in yellow-feathered roosters. Total testosterone levels were significantly higher in the FBP group than in the control group (2.80 nmol/L *vs.* 2.32 nmol/L, $P=0.03$). LH concentrations showed a tendency to increase in the FBP group (4.23 mIU/mL *vs.* 3.45 mIU/mL), although the difference was

not statistically significant ($P=0.21$). FSH levels rose from 5.21 IU/L in the control group to 6.23 IU/L in the FBP group, reaching statistical significance ($P=0.04$). These findings suggest that FBP supplementation may enhance testicular endocrine function, particularly by elevating testosterone and FSH levels.

3.7. Effects of FBP on autophagy-related protein expression

To assess the impact of FBP supplementation on testicular autophagy, protein expression levels of Beclin-1, ATG5, mTOR, and phosphorylated mTOR (p-mTOR) were evaluated using Western blotting. As shown in Figure 4, Beclin-1 and ATG5 expression levels were significantly upregulated in the FBP-treated group compared to the control group. Moreover, p-mTOR protein levels were reduced in the FBP group, while total mTOR expression remained unchanged.

Table 4. Effects of fermented bamboo powder on testicular morphometric and gonadal indices in roosters.

Parameters	Dietary treatments		<i>P</i> value
	Control	FBP	
Total cholesterol, mg/dL	120.00 $\pm$ 9.73	118.00 $\pm$ 8.67	0.32
Triglyceride, mg/dL	68.52 $\pm$ 8.23	45.20 $\pm$ 5.35*	0.04
Total protein, g/dL	4.23 $\pm$ 0.36	4.68 $\pm$ 0.41*	0.02
Uric acid, mg/dL	6.15 $\pm$ 0.72	5.90 $\pm$ 0.91	0.23
LDL, mg/dL	65.26 $\pm$ 6.02	52.42 $\pm$ 8.28	0.38

Data are expressed as mean $\pm$ SD ($n=10$ per group). Superscript asterisks (*) indicate significant differences between groups within the same row ($P<0.05$).

4. Discussion

The reproductive efficiency of roosters declines markedly with age, often leading to reduced fertility and productivity[7]. Natural bioactive compounds have shown promise in mitigating these effects; for example, ginger has been reported to restore male reproductive function in aging roosters[26]. In this study, FBP supplementation significantly improved testicular morphometry, including increased size of the left testis and width of the right testis, along with higher individual and total testis weights. Although the GSI and some right testis dimensions showed non-significant trends, they indicated a positive direction. These results were consistent with previous findings where folic acid improved reproductive performance in aged roosters[22], supporting the role of nutritional supplementation in enhancing reproductive health. The improved reproductive performance in the FBP group may also reflect FBP's nutritional support of accessory reproductive structures such as the vas deferens and epididymis, which are crucial for sperm maturation and transport.

The researcher selected parameters such as seminiferous epithelium height, testicular score, and seminiferous tubule diameter because they are direct indicators of reproductive health and fertility potential in roosters[27]. In the present study, dietary FBP supplementation significantly increased the seminiferous tubule diameter, seminiferous tubule area, seminiferous epithelial height, luminal area, and Johnsen's testicular scoring in yellow-feathered roosters, suggesting enhanced testicular development and improved spermatogenic activity. These findings were consistent with those of Ye *et al*[22], who also reported significant improvements in seminiferous epithelium height and testicular scores in aged breeder roosters following dietary intervention with FBP supplementation to evaluate its effect on reproductive health.

The present study demonstrates that dietary supplementation with FBP significantly upregulated the mRNA expression of key spermatogenesis-related genes such as *CREM*, *PCK2*, *DDX4*, *GDNF*, *SPAG6*, *SPAG16*, and *SPAG17* in the testes of roosters. These findings suggest that FBP may enhance essential biological processes critical for maintaining and promoting spermatogenic activity, thereby supporting improved male fertility. Each of these genes plays a distinct role in different stages of germ cell development. For instance, *CREM* acts as a master transcriptional regulator of post-meiotic germ cell differentiation and is essential for the transition from round to elongated spermatids. *PCK2*, a key mitochondrial enzyme in gluconeogenesis, contributes to energy metabolism, which is vital for sustaining the high energy demand of spermatogenic cells. *DDX4*, a germ cell-specific RNA helicase, regulates mRNA translation and stability, ensuring proper germ cell proliferation and meiotic progression. *GDNF* is indispensable for the maintenance and self-renewal of spermatogonial stem cells, thereby preserving

the foundation of continuous spermatogenesis. Similarly, *SPAG6*, *SPAG16*, and *SPAG17* encode sperm-associated proteins that are integral to flagellar structure and motility, directly influencing sperm quality and fertilization potential. These molecular outcomes align with and extend previous research emphasizing the positive effects of nutritional interventions on male reproductive performance. For instance, folic acid supplementation has been reported to enhance sperm concentration and motility in aged roosters by modulating spermatogenesis-related gene expression[28], while Alpha-Lipoic Acid has been shown to upregulate testis-expressed genes associated with germ cell development[29]. Moreover, energy provision has been reported to regulate testicular development and spermatogenesis by modulating differentially expressed genes[30]. A consistent theme across these studies, including the present findings, is that nutritional supplementation improves reproductive performance by enhancing cellular function, sustaining energy metabolism, and regulating gene expression within the testes.

In avian species, reproductive regulation is initiated by hypothalamic secretion of gonadotropin-releasing hormone (GnRH), which stimulates the release of LH and FSH. Given their roles in spermatogenesis, it was hypothesized that FBP supplementation would increase testosterone, LH, and FSH levels in roosters. However, while testosterone concentrations and FSH levels significantly rose in the FBP group, no statistically significant changes were observed in LH levels. These findings were consistent with Asl *et al*[31], who reported increased testosterone and FSH without changes in LH following polyunsaturated fatty acid (PUFA) supplementation, and with Vasankari *et al*[32], who noted similar outcomes in rats treated with a somatostatin analog. Conversely, Feng *et al*[33] reported simultaneous increases in all three hormones with PUFA-enriched diets in roosters, suggesting variability in hormonal responses based on age, species, or sampling protocols. Although LH levels in FBP-fed roosters were numerically higher than controls, the lack of statistical significance may also reflect methodological limitations, as hormone measurements were based on a single blood sample per rooster. Reproductive hormones such as LH are released in a pulsatile manner, and blood collection can induce stress, both of which may suppress LH secretion and influence the measured concentrations. Nevertheless, the trend toward higher LH likely contributed to enhanced testosterone production, as reflected by the positive association between LH concentration and testosterone levels. Overall, these results suggest that FBP improves male reproductive function predominantly by enhancing testicular steroidogenesis rather than robustly altering gonadotropin secretion.

Serum biochemical parameters are widely used in nutritional studies to assess the quality of feedstuffs or additives, as well as to provide valuable insights into the physiological and metabolic status of animals[34]. In the present study, FBP supplementation

led to a significant increase in total protein levels and a significant reduction in triglyceride concentrations. These findings suggest that FBP may enhance protein metabolism and improve lipid profiles, potentially contributing to better overall health and metabolic efficiency in roosters. Our results were consistent with the findings of Elsaid *et al*[35], who reported a significant decrease in triglyceride levels accompanied by a significant increase in total protein levels following dietary supplementation. Similarly, recent studies in broilers have shown that yeast supplementation significantly lowered triglyceride concentrations while increasing serum protein levels[36]. These parallels indicate that FBP, like other bioactive feed additives, may positively influence metabolic processes in poultry.

The present study demonstrates that dietary FBP supplementation significantly enhances autophagy-related protein expression in the testes of roosters, as evidenced by the upregulation of Beclin-1 and ATG5. These findings were consistent with previous studies showing that elevated autophagy supports germ cell renewal and protects against oxidative stress in aging testes[37,38]. Although total mTOR levels remained unchanged, phosphorylated mTOR (p-mTOR) decreased significantly in the FBP group. While mTOR is a known inhibitor of autophagy, recent evidence suggests a context-dependent role, where partial or dynamic activation of mTOR may coexist with active autophagy, particularly in metabolically active tissues like the testis[39]. This suggests that FBP modulates mTOR activity post-translationally, likely through energy-sensitive pathways such as AMPK-mTOR signaling, without disrupting mTOR's anabolic functions essential for spermatogenesis[40]. Similar to the effects of resveratrol in aged rat testes[41], FBP appears to create a favorable cellular environment by enhancing autophagic flux while preserving mTOR-driven growth processes.

However, this study has some limitations. The relatively short experimental period may not reflect the long-term effects of FBP on reproductive performance. In addition, only selected histological, biochemical, hormonal, and molecular indicators were evaluated, which may overlook other relevant reproductive or metabolic pathways. Future studies with longer durations and a broader range of physiological and molecular assessments are needed to fully understand the impact of FBP on poultry reproductive health.

In conclusion, FBP supplementation significantly improved reproductive performance in yellow-feathered roosters by enhancing testicular structure, upregulating spermatogenesis-related genes, and favorably modulating hormone and blood biochemical profiles. Its effect appears to involve an autophagy-mediated pathway, marked by increased expression of Beclin-1, ATG5, and p-mTOR. These findings suggest FBP is a promising functional feed additive for boosting male fertility in poultry.

Conflict of interest statement

The authors have no competing interests to declare.

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Data availability statement

All data generated or analyzed during this study are included in this article.

Authors' contributions

Rahmani Mohammad Malyar contributed to writing—original draft, writing—review & editing, visualization, validation, methodology, investigation, formal analysis, and data curation. Jiahao Sun and Shoura Hytham Elsaid contributed to writing—review & editing, investigation, formal analysis, and data curation. Mohammad Bayer Darmel contributed to writing—review & editing, methodology, and data curation. Yansen Li contributed to writing—review & editing, methodology, and resources. Wei Ding contributed to writing—review & editing, methodology, and data curation. Fangxiong Shi contributed to writing—review & editing, validation, supervision, resources, project administration, funding acquisition, and conceptualization. All authors have read and approved the final version of the manuscript.

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Fermented bamboo powder enhances testicular function and reproductive performance by modulating spermatogenesis, hormonal profiles, and autophagy in yellow–feathered roosters

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Supplementary Materials

Supplementary Table 1. Feed formulation of basal diet for the entire period of the experiment (d 1–77).

Items	Basal diet Starter phase (1-22 d)	Basal diet Growth phase (23-45 d)	Basal diet Finisher phase (46-77 d)
Corn	40.66	29.73	20.50
Wheat	20	40	50
DGYC	0	0	0
Soybean meal (43%)	22.31	5.73	2.92
Palm kernel meal	0	2	4
Sunflower kernel meal (35%)	3	5	8
Lard	0.00	0.00	6.03
Rapeseed meal (38%)	3	4	0
Corn gluten meal (60%)	4.0	5.0	4.4
Rice husk oil	2.31	4.22	0.00
Calcium bisphosphate	1.40	1.03	0.87
Limestone	1.18	1.12	1.13
Liquid methionine (88%)	0.14	0.23	0.15
Premix*	2	2	2

Total	100	100	100
Calculation of nutrients			
Metabolizable energy (MJ/kg)	285.6	300.8	307.6
Crude protein (%)	21.14	20.87	20.23
Crude fat (%)	4.048	4.410	4.530
Methionine (%)	0.486	0.764	0.491
Lysine (%)	1.097	1.364	1.130
Calcium (%)	0.989	0.962	0.970
Available phosphorus (%)	0.485	0.510	0.450

*premix provides the following nutrients (per kilogram of diet): choline, 1 g; enzyme complex, 300 mg; broiler multivitamin, 300 mg; glyceryl tributyrate, 300 mg; probiotics, 200 mg; organomineral, 400 mg; phytase, 200 mg; NaCl, 2.8 g; vitamin A, 9800 IU; vitamin D3, 2500 IU; vitamin E, 60 IU; vitamin K, 2.3 mg; vitamin B₁, 2 mg; vitamin B₂, 6 mg; vitamin B₆, 4 mg; vitamin B₁₂, 0.02 mg; nicotinic acid, 45 mg; pantothenic acid, 20 mg; folic acid, 1.25 mg; biotin, 0.02 mg; Fe, 75 mg; Cu, 8 mg; Mn, 75 mg; Zn 60 mg; Cu, 8 mg; Se, 0.3 mg; Co, 0.3 mg.

Supplementary Table 2. Analyzed composition of the fermented bamboo powder.

Testing item	Fermented bamboo powder
Moisture (%)	11.21
Crude protein (%)	17.07
Coarse fiber (%)	17.66
Crude fat (%)	3.48
Coarse ash content (%)	9.21
Acid soluble protein (%)	7.13
Acid washing lignin (%)	3.41
Calcium (%)	0.12
Total phosphorus (%)	0.02
Unknown substance (%)	18.81

Supplementary Table 3. Testis scoring (Johnsen score)

Score	Scoring criteria
5	Full spermatogenesis with complete and organized seminiferous tubules; abundant mature spermatozoa
4	Few or no spermatozoa, but spermatids are present
3	Few or no spermatids, but spermatocytes (primary or secondary) are present
2	Only spermatogonia are observed in the seminiferous epithelium
1	No germ cells present; tubules may be atrophic or contain only Sertoli cells