



RESEARCH ARTICLE

Impact of Fermented JUNCAO Feed on Health and Growth in Yellow-Feathered Broilers: Insights into Antioxidant and Immune Regulation

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ABSTRACT

JUNCAO (*Pennisetum giganteum*) has become a promising option for addressing livestock and poultry feed shortages due to its superior biological attributes contributing to economic benefits. This study assessed replacing traditional feed with fermented JUNCAO grass (FJCF) in yellow-feathered broiler birds. Two hundred 42-day-old male, yellow-feathered broilers were randomly assigned to control, and FJCF replacement ratios of 10%, 20%, and 40% groups to observe the effects of different replacement ratios. Results indicated that the 20% FJCF dietary group exhibited significantly superior average daily weight gain, survival rate, daily feed intake and feed conversion ratio (FCR) relative to control group ($P < 0.05$). The qPCR analysis of muscle tissues revealed that 20% and 40% FJCF supplementation significantly upregulated *LPL* gene in breast and *CAST* gene expression in leg muscle, respectively ($P < 0.05$). Addition of 20% FJCF dietary supplementation significantly enhanced the activity and ratios of $CD4^+/CD8^+$ T lymphocytes in chicken spleen, markedly higher than both 10% and 40% replacement groups ($P < 0.05$). Furthermore, FJCF supplementation significantly ($P < 0.05$) elevated serum levels of key cytokines (IFN- γ , IL-4, IL-2, TNF- α), immunoglobulins, and antioxidant factors. A dose-dependent increase in serum lysozyme activity was also observed. FJCF supplementation boosts humoral immunity in broilers without causing toxicity or inflammatory damage in immune organs (thymus, spleen, bursa), as confirmed by HE staining. Our study shows that 20% supplementation of FJCF promotes growth performance, enhances meat flavor, and immune response in yellow-feathered broilers by increasing splenic $CD4^+/CD8^+$ T lymphocyte ratios. The effects are dose-dependent, with 20% replacement being optimal. This study supports FJCF's use in poultry production.

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INTRODUCTION

To meet the growing demand for protein in an expanding global population, poultry has emerged as the most efficient livestock for production (McDevitt *et al.*, 2006) and combating the demand of protein efficiently. Chicken meat is rich in polyunsaturated fatty acids which are essential for human health particularly n-3 PUFAs (n-3 polyunsaturated fatty acids) (Ponte *et al.*, 2008). Maize and soybean, the principal protein sources, constitute 70% of traditional poultry feed worldwide, representing the most significant expense for poultry farmers (O'BRYAN *et al.*, 2014). However, the use of pesticides for cultivation of

maize and soybean, along with the extensive use of antibiotics in poultry feed, has led to challenges such as antibiotic resistance, (Xiao *et al.*, 2024) oxidative stress, and the deterioration of meat quality and flavor, ultimately reducing feed efficiency. This has indirectly increased the cost of disease treatment in both humans and poultry (Nhung *et al.*, 2017). In developing countries, the shortage of animal protein supplies necessitates the urgent exploration of alternative and novel feed sources (Franzuebbers and Martin 2022). In the post-pandemic era, the immune capacity of livestock, product quality, and food safety continue to constrain the healthy development of the poultry industry (Maqbool *et al.*, 2024). There is a

critical need for sustainable poultry models that lessen reliance on antibiotics, maize, and soy. Forage has emerged as a viable solution, capable of supplying essential protein while improving health and growth (Abouelezz *et al.*, 2012; Ibrahim *et al.*, 2023).

JUNCAO (*Pennisetum Giganteum*), known for its exceptional biological characteristics and associated economic benefits, has emerged as a promising solution to the shortage of livestock and poultry feed. It is now extensively cultivated in 31 provinces and autonomous regions in China, as well as in 87 countries globally (Shao *et al.*, 2025). Fermented JUNCAO, with reduced anti-nutritional factors, can partially replace commercial feed in chicken diets, leading to lower farming costs, improved growth performance, and enhanced immunity. This has demonstrated excellent practical results in numerous farms (Liu *et al.*, 2015).

The extract from JUNCAO-based fungus substrate, when added to broiler diets at a 5% ratio, improves digestibility and nutrient utilization, making it a novel feed additive that regulates immunity, antioxidant capacity, and growth performance in broilers (Omoor *et al.*, 2024). Two different studies demonstrated that incorporating 14% green grass into the diet of laying hens significantly enhances reproductive capacity while reduced the cholesterol contents (Puchajda-Skowrońska *et al.*, 2010). JUNCAO is rich in unsaturated fats and Ω 3 fatty acids, along with polyunsaturated to saturated fatty acids (Fan *et al.*, 2023). These components play a crucial role in reducing the risk of cardiovascular diseases and improving survival rates in chickens (Hu *et al.*, 2001; Ganji *et al.*, 2003). Additionally, the abundance of α -linolenic acid (ALA) and fat-soluble antioxidant compounds (such as vitamin E homologues and β -carotene) in JUNCAO (Fan *et al.*, 2023) significantly contributes to enhancing antioxidant capacity and immunity in poultry (Omoor *et al.*, 2024).

Enzymes abundant in JUNCAO, including cellulase, xylanase, laccase, and phytase, promote the degradation of JUNCAO cellulose in broilers, enhancing the absorption of proteins and vitamins (Baptista *et al.*, 2023). Lactic acid produced during JUNCAO fermentation inhibits the growth of other microorganisms while eliminating trypsin inhibitor and tannin activity, ensuring the feed suitability of fermented JUNCAO (Abdelnour *et al.*, 2018). Through transcriptome analysis, differential gene expression, and the identification of β -glucosidase hub genes, Zhou *et al.*, discovered that JUNCAO exhibits strong vitality under drought stress, making it suitable for cultivation in most regions worldwide (Zhou *et al.*, 2021). The JUNCAO Oasis 1 variety used in this experiment can yield up to 192,750 kg/ha, with leaves having a higher feed value than stems. Given JUNCAO's high nutritional value and environmental adaptability, it holds significant potential for reducing poultry farming feed costs through the partial substitution of commercial feed in the future (Huang *et al.*, 2024; Zhou *et al.*, 2021). Given the global cultivation and high nutritional value of JUNCAO, this study was designed to explore JUNCAO as cost-effective and economically efficient feed alternative. In the present study, we found that JUNCAO-fermented feed significantly enhanced growth performance, strengthened immune response, and boosted antioxidant levels in broilers, contributing to improved overall production.

MATERIALS AND METHODS

Ethical statement: Animal experiments complied with local laws and regulations and with the animal protection and utilization requirements of the Fujian Agriculture and Forestry University. Every effort was undertaken to reduce animal distress.

Humane endpoints: Birds were monitored twice daily for signs of severe distress (e.g., lethargy, inability to access feed/water, or labored breathing). Any animal meeting these criteria was immediately euthanized via cervical dislocation under anesthesia (isoflurane inhalation) to minimize suffering. No unexpected deaths occurred; all endpoints were planned sacrifices for sample collection at trial termination (Day 60). At the end, broilers were slaughtered according to standard procedures. Tissues were collected post-mortem for analysis.

Feed formulation: The fermented feed was prepared using JUNCAO Oasis No. 1 (Hayat *et al.*, 2022). Geese and chickens share some similar dietary requirements (Janiszewski *et al.*, 2021), and previous researchers have successfully applied goose dietary formulations to chicken trials (Omoor *et al.*, 2024). Therefore, the diet ratios in this experiment were formulated following some modifications, using the American Poultry Nutrition Standards (Council and Nutrition 1994).

Preparation of FJCF: JUNCAO was sourced from Zhen Nong JUNCAO Farm in Qingliang Town, Yongtai County, Fuzhou City, Fujian, China. JUNCAO plants were harvested when they reached a height of 1.5 meters (including stems and leaves), with the cutting point located 20 cm above ground level. Freshness was assessed by the ease of pinching the stem with fingers. The freshly harvested JUNCAO was bundled and cut using a specialized grass cutter into particles approximately 5 mm in diameter, with a stacking density of about 0.26 tons/m³ and a moisture content of approximately 55% (Wang *et al.*, 2019). The cut JUNCAO particles were then uniformly mixed with rice bran at a weight ratio of 8:2, placed in woven bags, sealed, and naturally fermented in a cool place for 24h at a controlled temperature between 28-40°C (Bolsen *et al.*, 1996). The FJCF was used within 24 hours of opening the bags.

Prior to feeding, the FJCF was converted to dry matter weight (13.5% moisture content) and, based on previous research experience, replaced maize and soybean at ratios of 10%, 20%, and 40% (Valdivié and Rodríguez 2016; Valdivié-Navarro *et al.*, 2020). An ANKOM 200i (USA) was used to measure the composition of JUNCAO-rice bran mixture after 24h of fermentation: DM (% FM) = 86.5, pH = 5.6, CP (g/kg DM) = 145, NDF (g/kg DM) = 653, ADF (g/kg DM) = 344, WSC (g/kg DM) = 13.4, LAB (log cfu/g FM) = 6.1. The detailed nutritional composition and combinations are given in Table 1.

Experimental animals and treatments: Two hundred 42-days old female yellow-feathered broilers (crossbred from traditional, yellow-feathered chickens and white-feathered broilers), healthy and of similar weight, were purchased from Aixin Family Farm in Chengfeng Town, Yongtai

County (Fuzhou, Fujian, China). The chickens (1090g-1150g) were randomly allocated to four groups, with five replicates each group and 10 birds per replicate. Each pen was approximately 1 m², using slatted flooring raised 0.5 m for ease of manure cleaning and ventilation. During the trial, birds had free access to feed and water, and good husbandry practices were observed. The chicken house temperature was maintained at 28-32°C with humidity around 50%. Fluorescent lights were installed above the chickens, providing 24-h illumination and ventilation. For current research period routine vaccinations were administered.

Table 1: Composition of experimental starter diets (dry matter basis) for 21- to 42-day broilers.

Components (%)	Fermented grass test groups (FG)			
	Control	10%	20%	40%
Corn powder	50.03	44.36	37.72	32.36
Soybean meal	31.02	26.77	23.59	19.04
Fermented grass	0	10	20	30
Oil	5	5	5	5
CaCO ₃	0.98	0.86	0.74	0.62
CaHPO ₄	1.87	1.86	1.85	1.85
Extruded-soybean	6.62	6.67	6.61	6.63
DL-methionine	0.16	0.17	0.18	0.19
Lysine	0.02	0.01	0.01	0.01
Premix*	4	4	4	4
NaCl	0.3	0.3	0.3	0.3
Nutrient contents				
ME, MJ/kg	3.23	3.24	3.13	3.6
CP, %	20.33	20.27	20.45	20.67
Ca, %	0.91	0.94	0.93	0.92
Ap, %	0.45	0.46	0.43	0.48
Lys, %	1.12	1.13	1.54	1.23
Met+Cys, %	0.76	0.77	0.74	0.78

The four experimental groups were as follows: 1) Control group, fed commercial feed; 2) 10% of maize and soybean replaced by fermented JUNCAO (FG); 3) 20% of maize and soybean replaced by FG; 4) 40% of maize and soybean replaced by FG. The trial was conducted on chickens aged 42 to 60 days. The dietary nutrition in this trial adhered to the Chinese Livestock Industry Standards (NY/T33-2004).

Measurement indicators and methods: Growth Performance: All chickens were weighed at 42 and 60 days of age to determine the initial body weight (IBW) and final body weight (FBW), measured in grams on an empty stomach in the morning. The average daily gain (ADG, g/bird/day) was calculated for each group. Daily feed intake and residual amounts were recorded to calculate the average daily feed intake (ADFI) for each group. FCR was then calculated by calculating the ratio of ADFI to ADG for each group.

Slaughter indices: At the end of the trial, 20 chickens (4 from each replicate) with weights close to the mean were selected from each group. Qualified personnel performed the slaughter according to standard procedures. Traditional dressing percentage (DP%, after bleeding and de-feathering), immune organ weights (thymus, spleen, bursa of Fabricius), and abdominal fat weight were measured, and relative weights (g/kg) were calculated (Banaszak *et al.*, 2021).

Serum biochemical indicators: At the end of the trial, 15 chickens (3 from each replicate) were randomly selected from each group for blood sampling (1.5 mL) from the wing

vein. Blood was collected in Eppendorf tubes and centrifuged at 3000 rpm for 5 min to separate serum, which was stored at -20°C for subsequent analysis. ELISA kits were used to detect cytokines (anti-inflammatory factors IL-4, IL-6, IL-10; pro-inflammatory factors IFN- γ , TNF- α , IL-1, IL-2, and IL-12) and immunoglobulins (IgA, IgM, IgG).

Antioxidant indicators: Chicken livers were washed with physiological saline, placed in cold isotonic saline solution, and stored at -70°C for later use. The frozen livers were thawed at 0°C, cut into small pieces, and mixed with chilled 10 mM potassium phosphate buffer (pH 7.4, 1.5% KCl (w/v) and 0.1 mM EDTA) with a ratio of 1:3 g/vol. Following centrifugation at 3000 rpm for 20 min the supernatant, was subjected to second centrifugation at 5000 rpm for 1 h to obtain the cytosolic fraction (Munteanu and Apetrei 2021). Antioxidant levels were then measured according to the method described previously (Kemball *et al.*, 2007).

PCR detection of LPL and CAST genes: Primer sequences for the LPL (lipoprotein lipase) and CAST (Calpastatin) genes of yellow-feathered broilers were designed using Premier 5.0, β -actin (GenBank NM_205282, NM_001034034.2), as detailed in Table 4. The primers were purchased by Wuhan Servicebio Engineering Co., Ltd. Five samples each of breast and leg muscle tissues were collected from sacrificed chickens in each group, with each sample weighing 100 mg. Total RNA was extracted by commercial kit, and RNA purity and concentration were determined via ultra-micro spectrophotometry, with acceptable OD260/280 ratios ranging between 1.8-2.0. Subsequently, cDNA was synthesized via reverse transcription according to the manufacturer's protocol (Roche).

PCR amplification was conducted in a 20 μ L reaction system comprising: 8 μ L RNase-free water, 10 μ L 2 \times T5 Master Mix, 0.5 μ L forward primer (10 μ mol/L), 0.5 μ L reverse primer (10 μ mol/L), and 1 μ L cDNA template (1260 ng/ μ L). The PCR program was adjusted as following, pre-denaturation at 95°C for 5 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 40 seconds. Using the synthesized cDNA as template, fluorescent quantitative PCR was performed. The qPCR reaction system comprised 20 μ L, specifically consisting of: 7.8 μ L RNase-free water, 0.8 μ L for each primer (F' and R) (10 μ mol/L each), 10 μ L universal SYBR Green premix solution, and 2 μ L cDNA template (140 ng/ μ L). The PCR program was designed as following, pre-denaturation at 95°C for 10 minutes; 40 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 40 seconds.

Isolation and detection of splenic CD4+ and CD8+ T lymphocytes: Five chickens were selected randomly from each group to sacrifice, and spleens were aseptically removed and thoroughly rinsed with phosphate-buffered saline (PBS). The spleens were cut into small fragments and placed on a cell strainer, where they were gently and slowly ground until completely dissociated and no visible tissue fragments remained. Subsequently, the strainer was rinsed with PBS to collect the splenic cell suspension. Equal volume of lymphocyte separation medium was slowly supplemented to

the prepared splenic suspension, mixed thoroughly, and centrifuged at 3000 rpm for 20 minutes. The white cloudy layer at the interface, containing splenic lymphocytes, was carefully aspirated. These cells were washed with PBS and centrifuged at 2500 rpm for 15 minutes. Following centrifugation, the cell pellet was collected, resuspended in PBS, and formulated into a final 1 mL cell suspension.

Subsequently, an antibody mixture containing anti-chicken CD3-FITC, anti-chicken CD4-PE, and anti-chicken CD8-APC antibodies (2 μ L each, Southern Biotech, Birmingham, USA) was added to the 1 mL splenic cell suspension. After incubation for 30 minutes at 4°C under light-protected conditions, the cell suspension was centrifuged at 1000 rpm for 5 minutes at 4°C. The supernatant was discarded, and the cells were resuspended in cell blocking solution. Finally, the differentiation levels of CD4⁺/CD8⁺ T cells were detected and analyzed using flow cytometry (Agilent, USA) (Kemball *et al.*, 2007).

Data were analyzed using a one-way ANOVA in SPSS, with a significance level set at $P < 0.05$. The homogeneity of variance was tested for all groups and was met. Post-hoc comparisons using the SNK test confirmed significant differences between group means ($P < 0.05$).

Histopathological analysis: On day 60, samples of the spleen, thymus, and bursa of Fabricius were harvested from five each group. The tissues were subsequently preserved in 4% paraformaldehyde and subjected to hematoxylin and eosin (HE) staining for histological examination.

RESULTS

Effects of FJCF on growth metrics of yellow-feathered broilers: The final body weight (FBW) and average daily gain (ADG) of broilers fed 10% and 20% FJCF were significantly higher than those of other experimental groups ($P < 0.05$) (Fig. 1). In contrast, the 40% group exhibited significantly lower values of weight gain than the other two

supplemented groups. The ADFI and FCR in the 20% and 40% FJCF groups were significantly different from each other ($P < 0.05$) and both were significantly higher than those in the control and 10% groups. However, the 20% group demonstrated ideal daily ADG and FCR, also the highest survival rate at 98%, which was significantly higher ($P < 0.05$) than in control and 40% groups.

Impact of FJCF on slaughter performance of yellow-feathered broilers: The effects of different proportions of FJCF on traditional dressing percentage and slaughter performance of birds are presented in Fig. 2. No significant changes ($P > 0.05$) between control and treatments were observed for relative weights (ratio to carcass weight) of carcass, abdominal fat, breast muscle, and leg muscle across all experimental groups. Similarly, no significant differences ($P > 0.05$) were found in the relative weights of spleen, thymus, and bursa of Fabricius. However, the 20% group showed a significantly higher relative weight of the gizzard compared to both the control and other experimental groups ($P < 0.05$).

Effect of FJCF on *LPL* and *CAST* gene expression: As shown in Fig. 3A and 3B, the expression of the *LPL* gene in breast exhibited a trend of initial increase followed by decrease with increasing proportions of FJCF in the diet. The 20% FJCF group showed significantly higher expression than control group ($P < 0.05$). However, in leg muscle tissue, the different dietary treatments did not affect *LPL* gene significantly ($P > 0.05$).

Furthermore, as illustrated in Fig. 3C and 3D, the addition of FJCF to the diet had no significant effect on *CAST* gene expression in breast muscle tissue ($P > 0.05$). Nevertheless, in leg muscle tissue, the treatment group with 20% FJCF significantly increased *CAST* gene expression, which was significantly higher than the control group ($P < 0.05$).

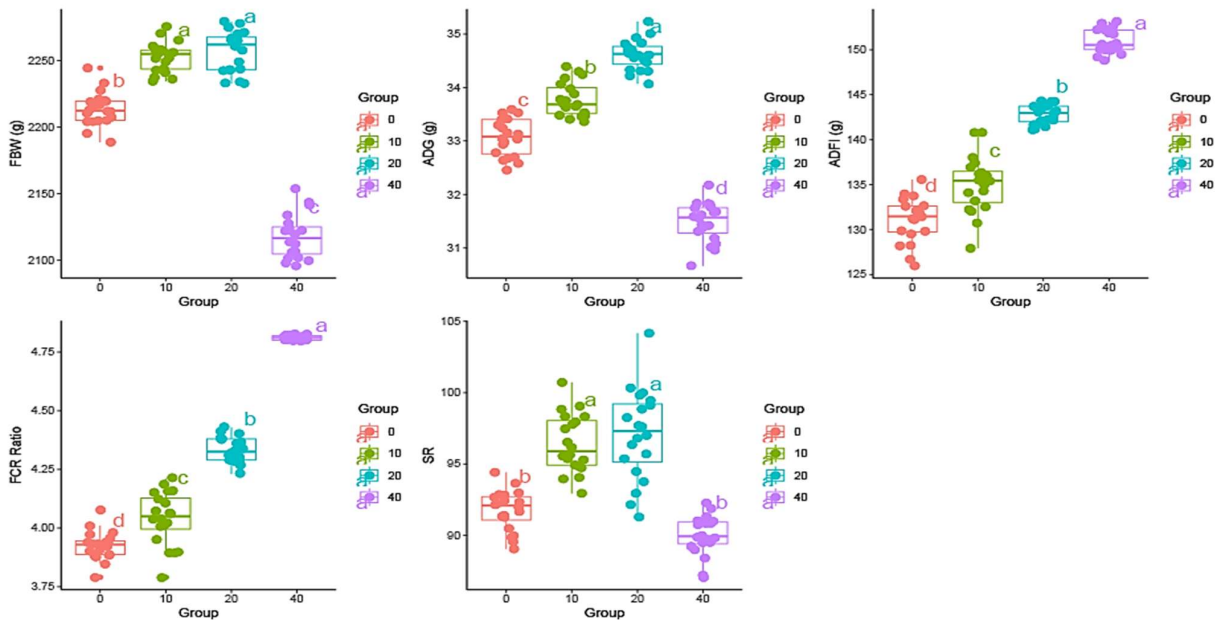


Fig. 1: Growth performance of broiler birds feed on various levels of fermented JUNCAO (60 days of age). Means with superscript letters (a–d) with in each row are significantly different at $P < 0.05$. FBW, final body weight; ADG, average daily gain; ADFI, average daily feed intake; FCR, feed conversion ratio; SR, survival rate.

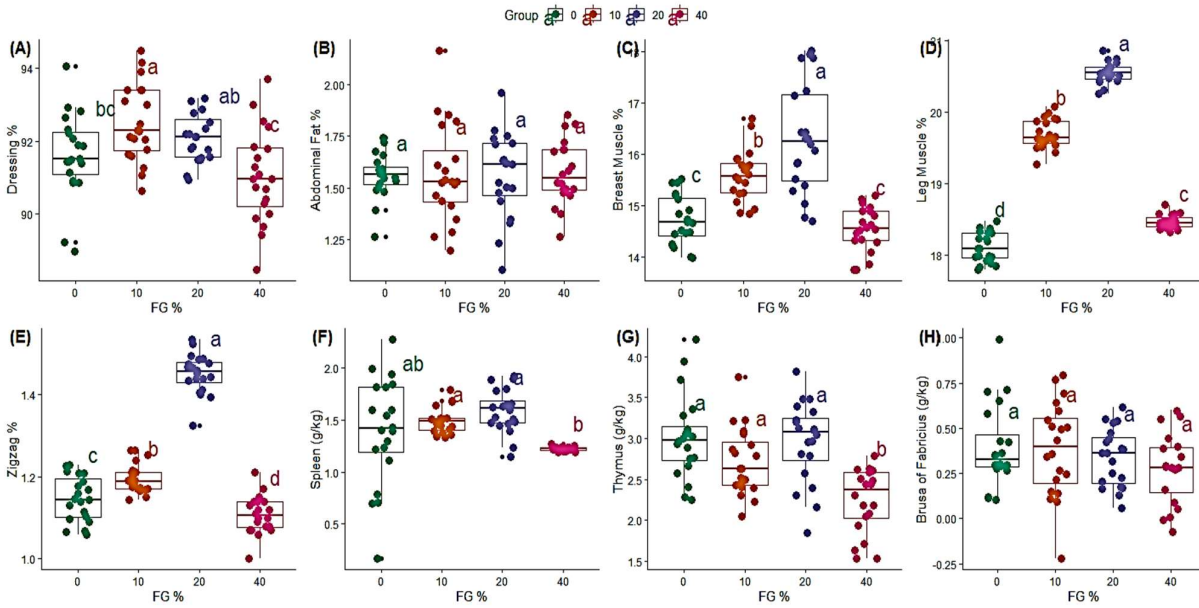


Fig. 2: Effects of varying dietary FG inclusion levels (0%, 10%, 20%, and 40%) on physiological and organ-specific parameters in broiler birds. (A) Dressing percentage, (B) Abdominal fat percentage, (C) Breast muscle percentage, (D) Leg muscle percentage, (E) Gizzard percentage, (F) Spleen index, (G) Thymus index, and (H) Bursa of Fabricius index. Different letters above the boxplots denote statistically significant differences between groups ($P < 0.05$).

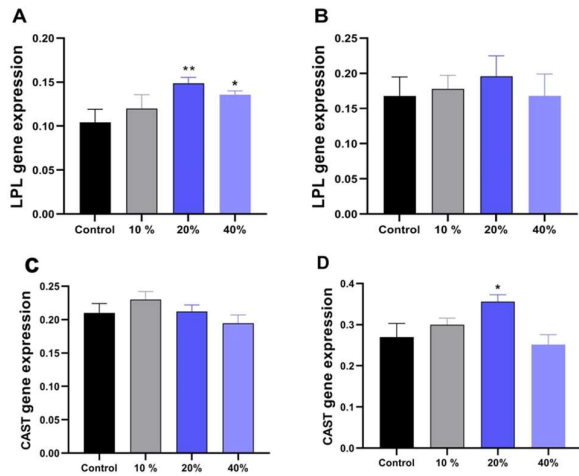


Fig. 3: Expression patterns of *LPL* and *CAST* genes in different tissues. (A, B) Expression of *LPL* gene in breast and leg muscles, respectively; (C, D) *CAST* gene expression breast and leg muscles, respectively.

Effects of FJCF on Inflammatory and Immune Responses in Yellow-Feathered Broilers: The immune parameters of the chickens are given in Table 2. In the 10% and 20% FJCF substitution groups, we observed that as the substitution ratio increased, the levels of IL-4, IL-6, and IL-10 in chicken serum increased significantly ($P < 0.05$). Concurrently, the levels of immunoglobulins IgG, IgA, and IgM also showed significant elevation ($P < 0.05$). However, in the 40% group, while anti-inflammatory factors increased, pro-inflammatory factors (IFN- γ , IL-1, IL-2, and IL-12) also exhibited a marked increase ($P < 0.05$). Serum lysozyme levels rose significantly ($P < 0.05$) with increasing proportions of FJCF substitution.

The serum antioxidant indices of chickens are shown in Table 3. The antioxidant factors in the experimental groups, including glutathione peroxidase (GSH-Px), superoxide dismutase (SOD), glutathione (GSH), catalase

(CAT), and malondialdehyde (MDA), as well as total antioxidant capacity (T-AOC), were all significantly higher than control group ($P < 0.05$), and positively related to the ratio of FJCF.

Table 2: Effect of various level of fermented JUNCAO on interferon- γ , inflammatory parameters (IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, and TNF- α), immunologic parameters (IgA, IgG, IgM), and orientalis lysozyme in serum of broilers (60 days of age).

Parameter	Control	10 % FG	20%FG	40% FG
IFN- γ pg/mL	645.324 \pm 18.70 ^a	675.39 \pm 15.18 ^b	794.66 \pm 22.27 ^b	1028.33 \pm 21.34 ^a
IL-1 μ g/mL	376.45 \pm 7.17 ^c	420.15 \pm 11.70 ^b	458.53 \pm 12.06 ^b	553.78 \pm 8.34 ^a
IL-2 μ g/mL	186.15 \pm 3.28 ^d	231.54 \pm 5.46 ^c	284.85 \pm 13.24 ^b	358.66 \pm 12.13 ^a
IL-4 μ g/mL	120.19 \pm 4.50 ^b	125.80 \pm 3.90 ^b	144.53 \pm 4.32 ^a	82.57 \pm 4.51 ^c
IL-6 μ g/mL	34.70 \pm 1.37 ^b	39.62 \pm 1.63 ^b	45.93 \pm 1.86 ^a	28.51 \pm 1.19 ^c
IL-10 μ g/mL	46.75 \pm 1.58 ^b	54.31 \pm 1.14 ^b	60.05 \pm 1.83 ^a	36.44 \pm 1.91 ^c
IL-12 μ g/mL	5.60 \pm 0.28 ^c	6.21 \pm 0.32 ^b	6.63 \pm 0.46 ^b	8.79 \pm 0.45 ^a
TNF- α	53.03 \pm 1.11 ^c	60.67 \pm 1.26 ^b	61.98 \pm 3.32 ^b	84.35 \pm 2.55 ^a
IgA μ g/mL	198.19 \pm 10.37 ^c	337.81 \pm 15.08 ^b	402.74 \pm 10.43 ^b	134.14 \pm 12.33 ^d
IgG μ g/mL	248.17 \pm 10.03 ^b	300.44 \pm 5.54 ^a	328.37 \pm 12.05 ^a	205.55 \pm 9.06 ^b
IgM μ g/mL	602.66 \pm 22.33 ^c	832.67 \pm 30.23 ^b	950.90 \pm 41.15 ^a	558.05 \pm 41.12 ^d
LYS U/L	3.97 \pm 0.11 ^d	4.56 \pm 0.21 ^c	6.05 \pm 0.27 ^b	7.17 \pm 0.32 ^a

Superscript letters (a-d) within each row show significant differences of mean values at $P < 0.05$.

Table 3: Effects of varying levels of fermented JUNCAO on antioxidant in broilers serum (60 days of age)

Parameters	Control	10 % FG	20% FG	40% FG
T-AOC	23.17 \pm 0.26 ^b	24.09 \pm 0.18 ^b	24.88 \pm 0.18 ^a	25.38 \pm 0.21 ^a
GSH-Px	50.23 \pm 1.60 ^c	56.16 \pm 1.32 ^b	63.47 \pm 1.44 ^b	75.38 \pm 1.71 ^a
SOD	9.15 \pm 0.23 ^d	11.67 \pm 0.63 ^c	13.28 \pm 0.57 ^b	16.43 \pm 0.32 ^a
GSH	0.96 \pm 0.01 ^b	1.01 \pm 0.01 ^{ab}	1.02 \pm 0.01 ^a	1.02 \pm 0.01 ^a
CAT	34.89 \pm 1.83 ^d	43.77 \pm 1.31 ^c	49.28 \pm 1.59 ^b	62.75 \pm 2.1 ^a
MDA	1.43 \pm 0.01 ^a	1.14 \pm 0.01 ^b	1.02 \pm 0.02 ^b	1.37 \pm 0.02 ^a

Table 4: LPL and CAST Gene Primer Sequences

Gene	Primer sequences (5'-3')	Length /bp
β -actinF	GAGAAATTGTGCGTGACATCA	152
β -actinR	CCTGAACCTCTCATTGCCA	
LPL-F	GTATGCTGATGCCCTATCC	108
LPL-R	GGCTTCTGAATCCCAATGC	
CAST-F	TGAGGGTAAACCTGTCAAGCC	192
CAST-R	GGTTGCTTTAGTGGTGACGGT	

Effect of FJCF on CD4⁺/CD8⁺ T lymphocyte differentiation in yellow-feathered broiler spleen: The differentiation levels of CD4⁺/CD8⁺ T lymphocytes in broiler spleens were assessed using flow cytometry, with results illustrated in Figure 4. After 18 days of replacing commercial feed with FJCF, the differentiation levels of CD4⁺/CD8⁺ T lymphocytes in the spleens of experimental animals were significantly elevated ($P < 0.05$). The 20% feed replacement group demonstrated the most significant induction of differentiation, followed by the 10% group and the 40% group (Fig. 4).

Further, the differentiation levels in the 10% ($p < 0.05$) and 20% ($P < 0.001$) groups were significantly enhanced compared to the control group. Moreover, in comparisons amongst the three treatment groups, the 20% group demonstrated significantly superior promotion of CD4⁺/CD8⁺ T lymphocyte differentiation compared to the control groups ($P < 0.001$). Compared to the 20% group, the CD4⁺/CD8⁺ ratio in the 40% group showed a significant decrease ($P < 0.01$), although it remained slightly higher than that of the control group ($P > 0.05$).

Histological analysis: HE stained immune organs of broiler bird's bursa of Fabricius, spleen, and thymus following the administration of FJCF showed in Fig. 5. Compared with the group fed commercial feed, there were no significant signs of toxicity or inflammatory infiltration observed in the thymus, spleen, and bursa of Fabricius in the groups receiving 10%, 20%, or 40% fermented feed.

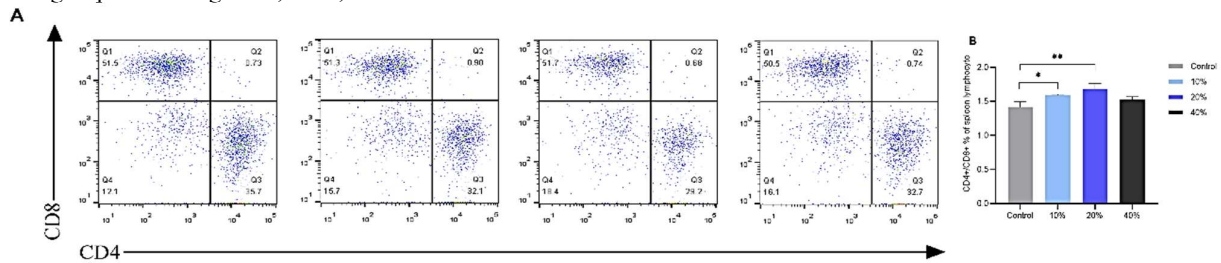


Fig. 4: The CD4⁺CD8⁺ T lymphocytes in the spleen. * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, all comparisons relative to the control group. The percentage of CD4⁺CD8⁺ spleen T lymphocytes of broiler birds administered diets containing 10%, 20%, and 40% FJCF for 60 days ($n=5$) (A). Representative flow cytometry plots depicting the gating strategy and proportions of CD4⁺CD8⁺ T lymphocytes are included for each experimental condition (B).

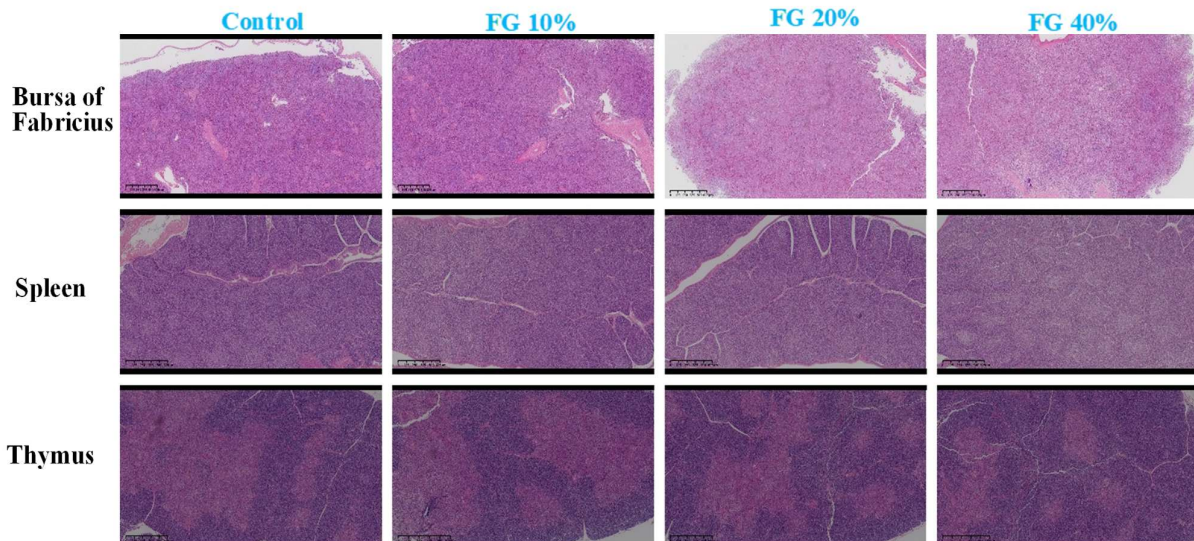


Fig. 5: HE histological analysis of bursa of Fabricius (top), spleen (middle) and thymus (bottom), from different groups on day 60.

These results indicate that substituting up to 40% of conventional feed with FJCF is safe for these immune organs. Consistent with the elevation of serum antibody levels, these findings further demonstrate that FJCF enhances the humoral immune response in broilers without inducing inflammatory damage to their immune organs (bursa of Fabricius, spleen, and thymus).

DISCUSSION

Modern poultry breeding for rapid growth and increased meat yield has increased feed cost and health issues like fat deposition reducing fertility and efficiency (Richards and Proszkowiec-Weglarz 2007). This study investigates the effects of partially substituting maize and soybean with FJCF effectively mitigates these issues by enhancing growth, immunity and antioxidant status, yellow-feathered broilers. We found that 20% FJCF substitution yielded optimal growth performance, improving final body weight and daily gain. This is likely due to Juncao's high protein content (20.6%) (Baptista *et al.*, 2023; Fan *et al.*, 2023), and fermentation-derived enzymes that improve fiber digestibility, aligning with findings that fresh forage improves gut health and nutrient utilization due to enhanced small intestine villus function (Zheng *et al.*, 2019), (Awad *et al.*, 2011). This fermented grass substitutional approach support significant feed cost reduction in poultry industry (Bouton 2007).

In many studies lymphoid tissues are often the primary focus (Müller *et al.*, 2003). In current study, we found that FJFC improved the immune function without affecting the relative weights of key immune organs (Thymus, Spleen and Bursa). This suggests that the immune modulation occurred without inducing organ stress or hypertrophy, a finding consistent with other forage inclusion (Omoor *et al.*, 2024). While free range rearing of poultry influenced these organs (Jin *et al.*, 2019).

Cytokines and interferons are crucial antiviral components of the innate immune system and serve as powerful defenses against avian viral infections (Anjum *et al.*, 2020; Anam *et al.*, 2021). The immunomodulatory effects were systemic: it was found that FJCF significantly elevated both pro-inflammatory (IFN- γ , IL-1, IL-2, IL-12, TNF- α) and anti-inflammatory (IL-6, IL-5, IL-10) cytokines, suggesting it helps to maintain a balanced and responsive Th1/Th2 immune state (Jeurissen *et al.*, 2000; Yoshimura *et al.*, 2007) Based on these observations, we propose that FG plays a role in regulating the immune state during periods of injury or disease and modulates Th1/Th2 cytokine secretion.

Furthermore, FJCF strengthened humoral and innate defenses, as evidenced by increased serum levels of immunoglobulins (IgA, IgM, IgG) and lysozyme (Woolf and Kerr 2006; Ibrahim *et al.*, 2023). In this study, chickens fed FG exhibited significantly higher levels of IgA, IgM, and IgG compared to the control group, with the 20% FG group showing the most pronounced effects. This suggests that FG enhances the regulation of humoral immunity in chickens.

Our observations indicate that serum lysozyme levels in chickens increased significantly with higher proportions of FG in their diet. This finding is consistent with previous studies by Doaa Ibrahim and elevated serum lysozyme levels in broilers fed solid-state fermented forage (Ibrahim *et al.*, 2023; Omoor *et al.*, 2024).

FJCF supplementation also improved the antioxidant system. We noticed a significant upregulation of hepatic antioxidant enzymes (T-AOC, SOD, GSH-Px, CAT) and a concurrent reduction in malondialdehyde (MDA), a marker of lipid peroxidation. These enzymes can act as biomarkers for oxidative stress (Olsvik *et al.*, 2005; Espinosa-Diez *et al.*, 2015). It can be stated that fermented forages can significantly bolster the endogenous defense against oxidative stress (Yin and Huang 2016; Omoor *et al.*, 2024). The rich polysaccharide content of fermented JUNCAO is a likely mechanism for this effect, as plant-derived polysaccharides are known for their antioxidant properties (Yuan *et al.*, 2015).

Dietary forage supplementation can enhance chicken splenic immune function by promoting T-cell proliferation and activation and regulating T-cell subpopulation ratios, ultimately improving humoral immunity in broilers (Chen *et al.*, 2003). At the cellular level, FJCF increased the proportions of splenic CD4⁺ and CD8⁺ T lymphocytes. The elevated CD4⁺/CD8⁺ ratio, which peaked at the 20% substitution, correlated with increased secretion of key cytokines like IL-2 and IFN- γ , indicating a coordinated activation of cellular immunity (Kemball *et al.*, 2007; Zhang and Zhao 2022). CD8⁺ T cells exert cellular immune functions through direct specific killing of infectious pathogens or tumor target cells (Zhang and Zhao 2022).

Previous studies have reported that dietary forage supplementation can significantly increase the ratio of CD4⁺ T to CD8⁺ T lymphocytes, thereby enhancing immune response capabilities in broilers (Zhang and Zhao 2022).

The flow cytometry results indicated that the CD4⁺/CD8⁺ ratio exhibited the highest level in the 20% group, the subsequent decline in this ratio in the 40% group suggests a threshold for optimal immune benefit, underscoring the importance of the inclusion rate. Beyond health metrics, FJCF improved the meat quality by influencing molecular markers. FJCF significantly upregulated the expression of *LPL* and *CAST* genes in muscle tissue is particularly noteworthy. *LPL* is involved in lipid metabolism and flavor development, while *CAST* regulates protein turnover and muscle growth (McDevitt *et al.*, 2006). This suggests that FJCF doesn't just support growth but actively promotes metabolic pathways associated with superior meat texture and flavor, a valuable finding for product quality.

Conclusions: In conclusion, our experimental results demonstrate that 20% FJCF is a promising strategy to enhance broiler immunity, antioxidant defense and potentially better meat quality. The efficacy of FJCF seems to be better than other fermented grasses, however it needs direct comparative studies. For further research, we should focus on long-term breeding studies to assess the cumulative effects of FJCF, and direct impact on farm profitability. We also need to focus its impact on gut microbiota composition, and elucidation of the precise molecular pathways through which it regulates gene expressions like *LPL* and *CAST*. This study provides a theoretical basis for the application of FJCF in livestock and poultry production, further establishing its potential for enhancing immune effects.

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Authors contribution: XZ and CBL performed experiments, collected and analyzed data and wrote and revised the manuscript. MUG, analyzed data, Re-write and edited the manuscript, WQ revised, conceived the study, participated in study design and coordination. All authors read and approved the final manuscript.

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