DOI:10.22059/IJVM.2024.369109.1005483 Iranian Journal of Veterinary Medicine Original Article

Online ISSN: 2252-0554

Fermented Feather Meal Improves the Antioxidant Status, Meat Quality, and Immune Response of Broilers

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Abstract

Background: The biodegradation of feathers is an effective alternative to other processing methods.

Objectives: This study investigated the effects of various hydrolyzed feather meals (HFM) on antioxidant status, meat quality, and immune response of broilers.

Methods: A total of 480-day-old Ross 308 male broilers were used for 42 days in a completely randomized design with eight treatments and five replicates (12 chicks/replicate) in the Research farm of Agriculture Faculty, Guilan University (Rasht, Iran) in October 2022. Experimental diets were: (1) a control diet (without feather meal; FM), (2), (3), (4), (5), (6), (7), and (8) all containing 4% raw FM (RFM), HFM by autoclave (Au-HFM), fermented FM (FFM) by *Bacillus licheniformis* (Bl-FFM), FFM by *Bacillus subtilis* (Bs-FFM), FFM by *Aspergillus niger* (An-

FFM), FFM by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger* (Co-FFM), and HFM by an enzyme (En-HFM), respectively.

Results: Results of the *in vitro* experiment showed that the 2,2-diphenyl-1-picrylhydrazl (DPPH) and 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging activity, as well as microorganisms count of FFM increased by increasing the fermentation time ($P \leq 0.05$). Also, the pH of FFM significantly decreased by increasing the fermentation time ($P \leq 0.05$). The DPPH and ABTS radical scavenging activity of FFM was higher than RFM, Au-HFM, and En-HFM ($P \leq 0.05$). Results of the *in vivo* experiment showed that the serum levels of total antioxidant capacity, glutathione peroxidase, superoxide dismutase, and catalase in broilers fed the FFM were higher than in control and other treatments ($P \leq 0.05$). Compared to control and other treatments, broilers fed FFM had lower meat malondialdehyde levels, higher meat pH, and higher water-holding capacity at 0, 7, and 14 d of the breast meat storage, as well as lower serum malondialdehyde levels ($P \leq 0.05$). Broilers fed FFM had higher antibody titer against Newcastle disease virus, lower heterophil count, and lower heterophil-to-lymphocyte ratio, as well as higher total anti-sheep red blood cells (anti-SRBC) titer, immunoglobulin G, and immunoglobulin M compared to control and other treatments ($P \leq 0.05$).

Conclusion: The fermentation of FM increased its antioxidant properties, which improved the body's anti-oxidative status and contributed to improving the meat quality and immune response of the broilers.

Keywords: Biodegradation; Broiler chicken; Feather meal; Fermentation; Radical scavenging.

1. Introduction

Reducing feed costs, reducing the cost of treatment, and increasing the quality of products in livestock production are the main challenges confronted by many researchers (Safari *et al*., 2016; Al-Muhammadawi & Jassim Hammoudi, 2022). Up to 70% of the total cost of commercial poultry production is spent on feed costs (Safari *et al*., 2016). As a result of the rising global price of soybean meal, the most commonly used source of protein in the diet formulation of poultry, competition between humans and animals for soybeans causes researchers to seek unconventional, alternative, or cheap sources of feed (Safari *et al*., 2016). Feathers make up about 5-10 percent of the live weight of poultry. The feathers, a great by-product of the poultry industry, contain 90% crude protein, mainly keratin. The disulfide bonds, hydrogen bonds, and hydrophobic properties of keratin result in its low solubilities and digestibility, hard to hydrolyze by enzymes, and limited bioavailability (Huang *et al*., 2022). Feathers are extremely resistant to common proteases and stable against different processes including thermo-chemical, autoclave, chemical, and mechanical (Prajapati *et al*., 2021). These intense processes denature and lose some amino acids, resulting in poor digestible feather meal (Huang *et al*., 2022). For example, autoclave conditions launch great amounts of waste gases, which include ammonia and sulfur dioxide, making them contaminating and unsustainable. Additionally, these methods produce non-nutritive amino acids such as lanthionine and lysinoalanine from Cys and Lys (Abdelmoteleb *et al*., 2023).

Among the different procedures, the biodegradation of feather keratin by keratinolytic microorganisms is feasible as an effective alternative to other methods, because of their quick growth and development, accessibility, large yield, cost-effective, sustainability, improved digestibility, and bioavailability (Abdelmoteleb *et al*., 2023). Additionally, the fermentation process increases the palatability of feed, improves growth performance, enhances beneficial gut

69 microbiota, and improves immunity in broilers (Ibrahim e*t al*., 2021). Microorganisms including
70 fungi, yeasts, actinomycetes, and *Bacillus* species can biodegrade feathers, which can utilize
71 feathers as the only source of both nitrogen and carbon (Pei *et al*., 2023). The main mechanisms
72 of feather keratin degradation in the fermentation process are as follows: (1) Breaking the
73 structural bonds and formation of the secondary structure of feather keratin: A decrease of the
74 disulfide link in feather keratin from cystine (-S-S-) to cysteine (-SH), causes the strongly bound
75 structure of keratin to alter to its biodegraded state (Abdelmoteleb *et al*., 2023). (2) Hydrolysis:
76 Macromolecules hydrolysis into soluble peptides and easily digestible small-size molecules
77 (Huang *et al*., 2022). (3) The keratin is completely degraded into bioavailable amino acids
78 (Abdelmoteleb *et al*., 2023). Feather keratin, as an ideal source of bioactive peptides and
79 oligopeptides, frequently has a variety of biological properties, including potent antibacterial,
80 immunomodulatory, and strong antioxidant properties (Kshetri *et al*., 2022). Feather is rich in
81 functional groups, such as -SH, -COOH, -OH, and -NH_2, which are present on keratin
82 polypeptide chains and lead to biological activity like strong antioxidant activity (Fakhfakh *et*
83 *al*., 2011). In this connection, from broiler feather hydrolysate, researchers extracted an
84 antioxidative octapeptide. Furthermore, it is well known that the intestinal mucosa's peptide
85 transport system facilitates the direct, energy-free absorption of small peptides (Huang *et al*.,
86 2019). It is well known that the quality of poultry meat is strongly related to their diet; therefore,
87 modulation of the animal feed could improve the quality and the nutritional value of their
88 products. Meat oxidation, water-holding capacity indicators, pH, and intramuscular fat all have a
89 direct impact on meat quality. Moreover, meat quality parameters like flavor, color, and nutrient
90 content are all impacted by oxidation (Safari *et al*., 2016). In the poultry industry, diseases rank
91 as the biggest economic challenge, after feed costs. Immunity and antioxidant levels are two key

metrics for assessing health, and nutrition and health are inextricably linked (Huang *et al*., 2022; Morovati *et al*., 2022; Mohammed Awad, 2023). Fermented feed improves not only humoral response (immunoglobulin contents, lysozyme activity, cytokine production) but also cell-mediated immunity and gastrointestinal health (Huang *et al*., 2022). Consequently, the goal of the current study was to investigate the impacts of various hydrolyzed feather meals (HFM) on antioxidant status, meat quality, and immune response in broilers.

2. Materials and methods

Preparation of various hydrolyzed FM

Preparation of FM: White broiler chicken feathers were collected from a slaughterhouse in the Rasht, Guilan province, Iran. The feathers were removed mechanically and washed (by hand in tap water), dirt-free, and then sun-dried. The material was then milled (MAKI-M-1282-1) and powdered (TS-2700) to a particle size of less than 0.5 mm on average. Powdered feathers were sterilized by autoclave (15 min, 121°C, 1.8 bar), then dried for 48 hours at 45°C in the sterile oven. Finally, one part of the dried FM was stored in the sterile bags for the raw feather meal (RFM). Meanwhile, the remainder was prepared for hydrolysis through an autoclave, a fermentation process, or an enzyme.

Fermentation of FM: The solid-state fermentation process was developed according to the methods of previous studies (Belewu *et al*., 2008; Jazi *et al*., 2017; Oluseun *et al*., 2016). The

steps for fermentation of FM were as follows: (1) *Bacillus licheniformis* (PTCC: 1595) and *Bacillus subtilis* (PTCC: 1720) bacteria, as well as *Aspergillus niger* (PTCC: 5154) fungi, were prepared from the Persian Type Culture Collection of Iranian Research Organization for Science and Technology (IROST). (2) *Bacillus licheniformis* and *Bacillus subtilis* were cultured on nutrient agar (Merck, Germany) and incubated for 48 h at 37°C and 30°C, respectively. *Aspergillus niger* was cultured on potato dextrose agar (PDA; Merck, Germany) and incubated for 72 h at 26°C. (3) One liter of distilled water was mixed with each kilogram of FM and 15 g of corn starch as a source of easily digestible carbohydrates and inoculated with either *Bacillus licheniformis* (10^9 colony-forming unit (CFU)/mL), *Bacillus subtilis* of (10^9 CFU/mL), and *Aspergillus niger* (10^9 spores/mL) or Co-fermented feather meal (Co-FFM) (10^3 CFU or spores/mL of each strain was used for Co-FFM) as fermented feather meal (FFM) treatments in a fermenter (10 L), with a one-way valve to the outflow of produced gases and prevent air entry (B. Braun Biostat B Fermenter/Bioreactor Controller, type 8840334), for 12 d at 30°C. (4) Finally, FFM was dried at 50°C for two days.

Autoclave hydrolysis: For this treatment, FM was hydrolyzed with an autoclave at 140°C and 2.5 bar steam pressure for 60 minutes (Wiradimadja *et al*., 2014).

Enzymatic hydrolysis: The enzyme used in this experiment was Cibenza IND900 (Novus International, Inc). Cibenza IND900 is a heat-resistant protease designed solely to increase the nutritional value of FM (Novus International, Inc). Cibenza IND900 has an enzyme activity of 65,000 U/g, according to the manufacturer. The steps for hydrolysis of FM by enzyme were as follows in compliance with the manufacturer's instructions: (1) 50 kg of raw feathers (50-60% moisture) were loaded into a batch. (2) 150 g of Cibenza IND900 was dissolved in 2 liters of water (Cibenza IND900 solution). (3) Cibenza IND900 solution was added to the feathers and

mixed well. (4) 50 kg of additional raw feathers (50-60% moisture) were added to the batch and mixed well again, and thoroughly mixed before being placed in the oven. (5) It was incubated at 55°C for 45 minutes. (6) The temperature was gradually increased to 120°C and pressure at 1.8 bar with agitation for 20 minutes by autoclave. (7) The HFM was dried at 45°C for 48 hours (Ajayi & Akoma, 2017).

In vitro experiment

Measurement of the physiochemical characteristics of fermented feather meals

For measurement of the pH value and microorganisms count, on 0, 4, 8, and 12 d of fermentation ten samples from each of FFM were taken. After homogenizing five grams of each sample in 45 milliliters of distilled water for one minute, the pH of the homogenate was measured using a digital pH meter (Inolab, Germany) (Safari *et al*., 2016). To determine the microorganisms count, 1 g of feed was added to 9 mL of sterile water, then homogenized. The supernatants were diluted 10-fold with buffered peptone water. Then 100 µL of supernatant were cultured on nutrient agar (Merck, Germany) and incubated for 48 h at 37°C and 30°C, for *Bacillus licheniformis* and *Bacillus subtilis* respectively. Also, *Aspergillus niger* was cultured on potato dextrose agar (PDA; Merck, Germany) and incubated for 72 h at 26°C (Belewu *et al*., 2008; Jazi *et al*., 2017; Oluseun *et al*., 2016).

Measurement of antioxidative activity of various HFM

Preparation of various HFM and RFM for antioxidative assay: Various HFM and RFM were prepared using the method described by Jeampakdee *et al*. (2020). Regarding these ten samples from each replicate of various HFM (on 0, 4, 8, and 12 d of fermentation for FFM) and RFM were milled and powdered to a particle size of 150-µm. Then, 0.5 g of each of the various HFM and RFM were mixed with 10 mL of 20-mmol phosphate buffer (pH=7.2), homogenized (High

Speed Homogenizer, D-500 Pro), and stirred (Magnetic Stirrer C-MAG HS) with 10 mL of 150-mmol NaCl overnight at 4˚C. Next, the samples were centrifuged for ten minutes at 10,000 × g at a temperature of 4˚C and the clear supernatants were collected. Supernatants were clarified by filtering through 0.45-µm filters (Whatman filter paper, GE, Buckinghamshire, UK) to remove the insoluble substrate. Then filtered supernatants were subsequently frozen at −20˚C for evaluating antioxidant potency by 2,2-diphenyl-1-picrylhydrazl (DPPH) and 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid (ABTS) radical scavenging activities assay. Samples were measured in triplicate in the laboratory for DPPH and ABTS radical scavenging activities.

DPPH radical scavenging activity assay: Methods described by Saisavoey *et al*. (2019) and Jeampakdee *et al*. (2020) were used to assay the DPPH radical scavenging activity. Briefly, a DPPH radical solution of 100 µmol in methanol was then added to each of the samples in the ratio of 1:4 (v/v), amounting to 80 µL of the sample with 320 µL of the DPPH radical solution. Then this mixture was incubated at 25˚C for 15 minutes in conditions of darkness. After 5 minutes of centrifugation at 12,500 × g, the solution was measured for absorbance at 517 nm (A_{517}) using a microplate reader. As the positive control, ascorbic acid (100 µg/mL) was used.

ABTS radical scavenging activity assay: Methods described by Saisavoey *et al*. (2019) and Jeampakdee *et al*. (2020) were employed in the assay for ABTS radical scavenging activity. Briefly, a 1:1 (v/v) mixture of potassium persulphate (2.45 mmol) and ABTS solution (7 mmol) had to be placed in total darkness and allowed to sit at 25˚C for 12 hours to produce ABTS cation radicals. Next, the cation radical solution ABTS was diluted to reach an absorbance of 0.7 ± 0.02 at 734 nm (A_{734}). Then, this solution was mixed with the test hydrolysate at a ratio of 1:30 (v/v), requiring 25 µL of the sample and 750 µL of the cation radical solution of ABTS. The

A734 was measured using a microplate reader after a 10-minute incubation period in the dark. As the positive control, ascorbic acid (100 µg/mL) was used.

Calculations of percentage inhibition: The radical scavenging percentage of samples was calculated (Saisavoey *et al*., 2019; Jeampakdee *et al*., 2020), as follows: [(Abs control – Abs blank) – (Abs sample – Abs background)/(Abs control – Abs blank)] × 100. Abs control: is the control's absorbance (no sample), Abs blank: demonstrates the absorbance of deionized water, Abs sample: denotes the various HFM or RFM absorbance, and Abs background: shows the level of color absorbance in the samples.

In vivo experiment

Experimental birds and diets

The current study was performed at the Poultry Research Station and Nutrition Laboratory of the University of Guilan, Rasht, Iran. A total of 480-one-day-old male broiler chickens (Ross 308) were obtained from the commercial hatchery, individually weighed, and allocated to a completely randomized design with eight dietary treatments and five replicates with 12 birds per replicate. The main housing conditions of chickens (chicken density, light regime, microclimate parameters, feeding, and drinking space) were identical for all groups and corresponded to the Ross 308 broilers raising guide for the year 2019. The chemical composition of raw feather meal (RFM), and various hydrolyzed feather meals (HFM) were analyzed and used for diet formulation. All diets were formulated according to the Ross 308 broiler chicken nutrients requirement guideline. The following were the experimental treatments: (1) Control diet (based on corn and soybean meal, without feather meal; FM), (2) Diet containing 4% RFM, (3) Diet containing 4% HFM by autoclave (Au-HFM), (4) Diet containing 4% fermented feather meal (FFM) by *Bacillus licheniformis* (Bl-FFM), (5) Diet containing 4% FFM by *Bacillus subtilis* (Bs-

205 FFM), (6) Diet containing 4% FFM by *Aspergillus niger* (An-FFM), (7) Diet containing 4%
206 FFM by *Bacillus licheniformis*+*Bacillus subtilis*+*Aspergillus niger* (Co-FFM), and (8) Diet
207 containing 4% HFM by an enzyme (En-HFM). The ingredients and chemical composition of the
208 starter (1–10 days), grower (11–24 days), and finisher (25–42 days) diets are presented in Table
209 1, Table 2, and Table 3 respectively. All diets were fed in the mash form, and the birds were
210 provided *ad libitum* access to feed and water during the study. The experiment lasted for 42 days.
211 **Serum antioxidant capacity**
212 At d 42 of the experiment, from each replicate, 3 birds were selected randomly, and 4 h after
213 feed withdrawal from the brachial vein, blood samples were taken. Then blood samples were
214 centrifuged at 3000 × g for 15 minutes, and collected serum samples were kept at -20˚C for
215 further analysis (Hosseinian *et al*., 2021; Xu *et al*., 2022). Finally, the serum level of
216 malondialdehyde (MDA), as well as activities of superoxide dismutase (SOD), catalase (CAT),
217 glutathione peroxidase (GPX), and total antioxidant capacity (TAC) of serum were assessed
218 using commercial kits (Kushanzist Azma- Parseh, Tehran, Iran), based on the manufacturer's
219 instructions. Samples were analyzed in triplicate in the laboratory for serum antioxidant capacity.
220 **Meat quality measurements**
221 At the end of the study (d 42), three birds from each pen were slaughtered and the breast meat
222 samples were individually sliced, washed in a solution of 0.9% NaCl, vacuum packaged in
223 plastic bags, and kept frozen at -20°C for 0 (immediately after sampling and before freezing), 7,
224 and 14 days to assess the meat quality characteristics. Samples were tested in triplicate in the
225 laboratory for meat quality measurements.
226 **Lipid oxidation:** After dilution of 1 g of breast meat sample with 9 mL of ice-cold phosphate-
227 buffered saline (pH = 7.2), homogenization and centrifugation at 9000 × g for 15 minutes at 4°C

were used to obtain muscle homogenates (Alahyaribeik *et al*., 2022). To measure MDA, the clear supernatants of the homogenates of breast meat were poured into vials. The MDA was determined using corresponding diagnostic kits (Kushanzist Azma- Parseh, Tehran, Iran) in compliance with the manufacturer's instructions.

pH: After homogenizing the five grams of breast meat for one minute in 45 milliliters of distilled water, the pH of the homogenate was measured using a digital pH meter (Inolab, Germany) calibrated at pH 4.0 and pH 7.0 (Safari *et al*., 2016; Partovi *et al*., 2021).

Water-holding capacity (WHC): The method of Aristides *et al*. (2018) was followed (with small modifications) to determine the WHC. Samples of meat were taken from the same location in the breast muscle and cut into 15.0-g (wet weight) cubes. First, samples were gently sandwiched between two sheets of filter paper (Whatman Filter Paper, No 2, 150 mm circle, 1002-150). Following a 5-minute exposure to a 35 kg force using a pressure instrument (Guangzhou Runhu Instrument Co, China), samples were weighed again. Finally, the percentage of WHC was determined according to the following equation:

WHC (%) = 100 – [((initial weight – final weight)/initial weight)) × 100].

Immune responses

Anti-sheep red blood cells (anti-SRBC) antibody assay: In the current study, as methods described by Rafat Khafar *et al*. (2019) and Hosseini-Vashan and Piray (2021) non-pathogenic antigens of sheep red blood cells (SRBC) were used to measure the broiler chickens' humoral immune response. The SRBC was collected and diluted in phosphate buffer saline (PBS; pH 7.5) to provide a 10% (vol/vol) suspension. At 28 and 35 d of age, 1 mL 10% suspension SRBC was intravenously administrated to the right wing of three birds per replicate. Then, 7 days after the first and second injections (at 35 and 42 d of age) the same broilers were bled through brachial

venipuncture, and 3 mL samples were collected for primary and secondary antibody responses, respectively. Blood samples were centrifuged for 15 minutes (at 2500 × g) at 4˚C and the obtained serum was kept at -20˚C for further analysis. In the current study, samples were assayed for total anti-SRBC antibody, serum levels of immunoglobulin G (IgG) and immunoglobulin M (IgM) by hemagglutination assay (HA) test, according to the method as previously explained by Rafat Khafar *et al*. (2019). Finally, antibody titers were reported as $\log_2$ of the reciprocal of the highest dilution giving visible agglutination. Samples were tested in triplicate in the laboratory for anti-SRBC antibody assay.

Anti-Newcastle disease virus antibody assay: In our study, to assay the humoral immune responses of broilers antigens Newcastle disease virus (NDV), all the birds were subcutaneously vaccinated in the back of the neck against Newcastle-influenza (H9N2 subtype) with 0.3 ml per chick on 8 d of age. In addition, birds were orally vaccinated against Newcastle disease (Lasota) on 18 d of age. To measure antibody titers against NDV on day 24 and 42, blood samples were taken from the brachial vein of three broilers from each replicate, and antibody titers production response against NDV were assayed using the hemagglutination inhibition (HI) method (Hosseini-Vashan & Piray, 2021). Samples were measured in triplicate in the laboratory. Finally, the HI antibody titers were expressed as $\log_2$ of the reciprocal of the highest dilution giving visible agglutination.

Heterophile (H) and lymphocyte (L) count: In the present study, to measure the immune responses of broilers on d 42, blood samples were taken from the brachial vein from three same selected birds per replicate in heparinized tubes (containing heparin to avoid blood clot formation). To acquire heterophile (H) and lymphocyte (L) count and H/L ratio, 1 drop of each blood sample was smeared on each of the three glass slides. Then May-Grunwald-Giemsa

staining was used to prepare blood smears. Then by counting 100 leukocytes on each slide, the H and L count, as well as the H/ L ratio were determined for each broiler (Jazi *et al*., 2019).

Statistical analysis

Levene's test and the Shapiro-Wilk test were used for variance homogeneity and normality of distributions respectively. Data were analyzed in a completely randomized design using the GLM procedures of SAS software (SAS, 2009). Significant differences among treatment means were determined using Tukey's multiple-range tests. Significance was based on $P \leq 0.05$.

3. Results

In vitro experiment

DPPH and ABTS radical scavenging activity assay

As shown in Table 4, the DPPH and ABTS radical scavenging activity, as well as microorganisms count of FFM significantly increased by increasing the fermentation time ($P \leq 0.05$). Also, the pH value of FFM significantly decreased by increasing the fermentation time ($P \leq 0.05$). As illustrated in Table 5, the DPPH and ABTS radical scavenging activity of FFM was significantly higher than RFM, Au-HFM, and En-HFM ($P \leq 0.05$).

In vivo experiment

Antioxidant enzymes

296 As presented in Table 6, serum levels of TAC, GPX, SOD, and CAT in broilers fed the FFM diet
297 were significantly higher than those fed the control, RFM, Au-HFM, and En-HFM diets ($P \leq$
298 0.05). Birds on the En-HFM diet had higher GPX, and SOD levels compared to the control and
299 RFM ($P \leq 0.05$). Broilers fed Au-HFM diet had higher GPX, and SOD levels compared to the
300 control ($P \leq 0.05$).

301 **Meat quality and serum MDA**

302 Table 7 presents the effects of various HFM on pH, WHC, and MDA in breast meat, as well as
303 serum MDA concentrations in broilers. The results showed that at 0 d of breast meat storage,
304 broilers fed En-HFM had a lower breast meat MDA compared to the control ($P \leq 0.05$), while
305 those fed diet containing FFM had the lowest level of breast meat MDA compared to the control,
306 En-HFM and other treatments ($P \leq 0.05$). Compared with the control and other treatments,
307 broilers fed FFM had lower breast meat MDA levels at 7 and 14 d of storage, as well as lower
308 serum MDA levels ($P \leq 0.05$). Broilers fed FFM had higher breast meat pH, and WHC at 0, 7,
309 and 14 d of storage compared with the control and other treatments ($P \leq 0.05$).

310 **Immune response**

311 Effects of various HFM on antibody response against SRBC, NDV, heterophil, and lymphocyte
312 in broilers are shown in Table 8. At 35 d broilers fed FFM had higher total anti-SRBC, IgG, and
313 IgM titers compared to the control and other treatments ($P \leq 0.05$). At 42 d birds on the En-HFM
314 diet had a higher total anti-SRBC titer compared to the control ($P \leq 0.05$), while those fed with
315 FFM had the highest total anti-SRBC titer compared to the control, En-HFM and other groups (P
316 ≤ 0.05). At 42 d broilers fed FFM had higher IgG, and IgM titers compared to the control and
317 other treatments ($P \leq 0.05$). Broilers fed FFM had higher antibody titer against NDV at both 24
318 and 42 d compared to the control and other treatments ($P \leq 0.05$). Birds on the FFM diet had

lower heterophil count, and lower heterophil-to-lymphocyte ratio compared to the control and other treatments ($P \leq 0.05$). No significant changes were observed in lymphocyte count among different treatments ($P > 0.05$).

4. Discussion

In vitro experiment

The physiochemical characteristics of fermented feather meals

The fermentation process results in the growth of a large number of fermenter microorganisms, which produce a high amount of some organic acids, which leads to a reduction in the pH of fermented feed (Engberg *et al*., 2009). This is consistent with the observation from the current study which exhibited a decrease in pH and an increase in microorganisms count by increasing the fermentation time.

DPPH and ABTS radical scavenging activity assay

In the current study, the in vitro antioxidant capacity of various HFM was assessed using the DPPH and ABTS assay. The results of the current study are in agreement with the findings of the previous studies, which demonstrated that fermented feather, (Callegaro *et al*., 2018; Bezus *et al*., 2021; Prajapati *et al*., 2021; Kshetri *et al*., 2022; Abdelmoteleb *et al*., 2023; Pei *et al*., 2023;) as well as oligopeptides and peptides produced from fermented feather (Huang *et al*., 2022) had a significantly higher antioxidant activity with DPPH and ABTS radical scavenging activity than control and RFM. The DPPH is a strong free radical that is scavenged when it comes into contact with a proton-donating material, such as an antioxidant, and its purple color changes to yellow, resulting in a decrease in absorbance (Ben Hamad Bouhamed *et al*., 2020). Various mechanisms, such as radical scavenging, chelation or reduction of metal ions, or a combination of these processes, can give protein hydrolysates their antioxidant properties. In the DPPH assay, the

342 latter becomes stable when an antioxidant transfers a hydrogen atom to a DPPH radical
343 (Callegaro *et al*., 2018). An electron-donating (antioxidant) molecule decreases the pre-formed
344 cation ($ABTS^{*+}$) to ABTS in the ABTS radical-scavenging assay, a decolorization technique that
345 results in reduced absorbance (Callegaro *et al*., 2018). According to reports, electron, and
346 hydrogen atom transfer are antioxidant mechanisms that both can be reflected by the ABTS
347 method (Callegaro *et al*., 2018). Therefore, loss of absorbance reflects the antioxidant activity of
348 the subject compound.
349 Studies have shown that the length of the peptide chain, molecular weight, and amino acid
350 sequence are connected to the antioxidant potential and scavenging ability of DPPH and ABTS
351 free radicals (Pei *et al*., 2023). Feather keratin hydrolyzed by microbial contains low molecular
352 weight free amino acids and small peptides that donate electrons to free radicals to create more
353 stable products (Ben Hamad Bouhamed *et al*., 2020). The low molecular weight of peptides can
354 increase their ability to interact more readily with the radicals, as a result, increasing antioxidant
355 activity and hindering the process of oxidization (Jeampakdee *et al*., 2020). As part of the
356 precursor protein, these peptides are inactive; they only become active when they are released
357 via hydrolytic cleavage and converted to bioactive peptides (Callegaro *et al*., 2018). Since
358 aromatic and hydrophobic amino acids promote interactions with DPPH and ABTS, there is a
359 tendency in protein hydrolysis to increase ionizable groups and produce hydrophobic and/or
360 aromatic groups, which have a high degree of antioxidant potential (Callegaro *et al*., 2018; Pei *et*
361 *al*., 2023). This is due to the peptide sequences' hydrophobic amino acid residues can improve
362 peptide solubility at the water-lipid interface, enabling more interaction with any radical species
363 that may be present (Callegaro *et al*., 2018; Pei *et al*., 2023). About 50% of the amino acid
364 residues in feather keratins' sequences are hydrophobic and/or aromatic (Pei *et al*., 2023). This

365 explains why unfermented FM achieves DPPH and ABTS radical scavenging activity. The
366 arrangement of the amino acids within the peptide sequence is one element that influences the
367 antioxidant activity of peptides (Pei *et al*., 2023). For example, His located at the C-terminal
368 functions as an efficient various radicals scavenger, whereas His located at the N-terminal
369 functions as an efficient chelator of metal ions (Jeampakdee *et al*., 2020). In the meantime,
370 tripeptides with Tyr or Trp at the C-terminal function as potent radical scavengers (Jeampakdee
371 *et al*., 2020). Diverse proteases, which are highly active across a wide pH range and primarily
372 classified as serine proteases that cleave peptide bonds near aromatic and/or hydrophobic
373 residues, are produced by bacteria and fungi during their growth on feather media (Pei *et al*.,
374 2023). In addition, as a result of microbial fermentation breaking down the feather keratin's
375 disulfide bonds, cysteine is created which has strong antioxidant properties. Since feather
376 peptide's cysteine-SH is a potent hydrogen donor to free radicals, cysteine has antioxidative
377 activity (Prajapati *et al*., 2021). Sulfenic acid (–SOH), and sulfhydryl (–SH) are yet another
378 prime antioxidant in feather keratin hydrolysate, which are produced when the feather is under
379 microbial fermentation process (Prajapati *et al*., 2021). Reductones which are present in
380 fermented feathers, have antioxidant activity due to the radical chain breaking by the donation of
381 a hydrogen atom. Free radicals can react with hydrogen or electron-donating amino acids and
382 peptides to be stabilized (Bezus *et al*., 2021).

383 **In vivo experiment**

384 **Antioxidant enzymes**

385 The quick induction of protective antioxidant enzymes is necessary for a cell to be able to
386 maintain functional homeostasis. The GPX, SOD, and CAT enzymes, which make up the first
387 barrier in the antioxidant defense system, are crucial in protecting cells and tissues from the

388 damaging effects of free radicals via scavenging oxygen free radicals by a chain reaction
389 mechanism (Xu et al., 2022; Obaid Saleh et al., 2022). The dismutation of superoxide anions to
390 hydrogen peroxide, the breakdown of H_2O_2 and hydroperoxides derived from unsaturated fatty
391 acids at the expense of reduced glutathione, and the conversion of H_2O_2 into water are all
392 catalyzed by the SOD, GPX, and CAT (Liu *et al*., 2020). Our findings are similar to the previous
393 studies, which pointed out that FFM and other fermented feeds increased the content of TAC,
394 GPX, SOD, and CAT in either serum or muscle and liver of broilers (Liu *et al*., 2020; Ibrahim *et*
395 *al*., 2021; Xu *et al*., 2022) piglets (Gu *et al*., 2021) and laying hen chicks (Zhu *et al*., 2020).
396 There are several potential methods by which FFM enhances the broiler's antioxidant status. (1)
397 The increased antioxidant capacity of the fermented feed can be attributed to the production of
398 lactic acid, small and bioactive peptides, and antioxidant vitamins during fermentation
399 (Sugiharto *et al*., 2019). (2) Microbial enzymes hydrolyze phenolic compounds such as phenolic
400 glycosides which are produced by microorganisms in the fermentation process and release free
401 aglycones, which have the potential for high antioxidant activity (Ibrahim *et al*., 2021). (3)
402 Keratinase produced by microorganisms can enhance the digestibility of minerals (Xu *et al*.,
403 2022), which might improve the utilization of selenium. The activity of GPX as a selenium-
404 dependent enzyme, could be improved by increasing selenium utilization (Xu *et al*., 2022). (4)
405 As well as keratinase of microorganisms can improve the hydrolysis of the proteins containing
406 cysteine to release cysteine, which is a precursor of glutathione, thus increasing the body's
407 synthesis of glutathione (Huang *et al*., 2022).

408 **Meat quality**

409 **pH**

Our outcomes are in line with the results of the researchers, who reported that fermented feeds including FFM significantly increased the pH value of the breast and thigh meat of broilers (Liu *et al*., 2020). The pH value of breast meat in broilers fed FFM was higher than the broilers from the control and unfermented FM groups; which indicates a positive correlation between strong antioxidant capacity and higher meat pH in broilers. The postmortem conversion of muscle glycogen to lactic acid primarily determines the pH value, one of the most significant indices of meat quality. In other words, the pH value directly reflects the meat acidity (Xu *et al*., 2022). Normally, the muscle cells in a broiler chicken that has been exsanguinated and subsequently suffer hypoxia eventually have to turn to anaerobic glycolysis to maintain metabolic activities. This is the only energy source available to the postmortem muscles (Aristides *et al*., 2018). Under this condition, body glycogen stores are depleted as they are converted to energy, and lactic acid, thereby reducing pH (Aristides *et al*., 2018). However, an antioxidant can neutralize the cation H^+ by direct reduction via electron donation and prevent reducing pH, and acidification of the meat (Callegaro *et al*., 2018). The pH decrease leads to meat myofibrillar protein denaturation, which impairs the proteins' ability to hold onto water inside the cell, tenderness, and color, thereby creating economic losses by decreasing carcass quality and reducing production efficiency in the poultry industry (Aristides *et al*., 2018; Majidi et al., 2023).

Water-holding capacity

In line with the results of the current study, other studies also have shown that fermented feed increased the WHC in muscles including the breast and thigh of broilers (Liu *et al*., 2020; Xie *et al*., 2021). In the present study, the results showed that WHC was consistent with the pH values. The improvement of pH and WHC of muscle can be related to the enhanced antioxidative status of broilers through feeding FFM. Loss of soluble nutrients, poor flavor, and drier, as well as

tougher meat can result from increased liquid outflow in muscles with low WHC (Xie *et al*., 2021). The inverse correlation between meat moisture and intramuscular fat, which is directly related to meat juiciness, could be the cause of the elevated WHC in muscle meat (Liu *et al*., 2020; Xie *et al*., 2021). Since unsaturated fatty acids make up the cell membrane, oxidation status, and meat WHC are closely associated. The fluidity and structure of the membrane are specifically affected by the oxidation of these fatty acids (Xie *et al*., 2021). Muscle WHC is reduced as a result of altered cell permeability due to the oxidation of the membrane phospholipids. High WHC in meat indicates low water loss, which can benefit meat processing, meat value, and meat appearance for consumers (Liu *et al*., 2020). The higher levels of antioxidants in broiler meat, which demonstrated potent free radical scavenging activity and decreased oxidatively-induced conformational changes and myofibrillar protein fragmentation, may be connected to the higher WHC in the meat (Xie *et al*., 2021). Furthermore, the denaturation of sarcoplasmic proteins to myofibrils may affect WHC in meat (Liu *et al*., 2020).

MDA of muscle and serum

In comparison to other meats, chicken meat has a comparatively high concentration of polyunsaturated fatty acids, such as the important n-3 fatty acids, and is more susceptible to free radical damage (Safari *et al*., 2016). In the current study, feeding FFM contributed to improved broiler meat quality, which was reflected by the reduction in meat postmortem MDA content. These findings are consistent with other research, which found that FFM or other fermented feeds decreased MDA amount in the serum (Drażbo *et al*., 2018; Gu *et al*., 2021; Xu *et al*., 2021; Elbaz *et al*., 2023), and muscle including thigh and breast of broilers, egg yolk homogenate, turkeys, and piglets (Aristides *et al*., 2018; Ibrahim *et al*., 2021; Alahyaribeik *et al*., 2022; Xu *et al*., 2022). The one of the important products of lipid peroxidation, known as MDA, indicates the

456 degree of lipid peroxidation and the generation of free radicals by reactive oxygen species (Safari
457 *et al*., 2016; Gholipour-Shoshod *et al*., 2023; Al-Abdaly *et al*., 2023). Also, lipid peroxidation
458 causes damage to the integrity of the muscle cell membrane, which can seriously cause meat to
459 lose nutrients and exudate (Safari *et al*., 2016). Moreover, reactive oxygen species harm
460 biological macromolecules such as proteins and nucleic acids. Lipid peroxidation produces
461 MDA, which can be incorporated into proteins through interactions with biomolecules. This can
462 lead to the generation of carbonyl derivatives, which have cytotoxic and genotoxic impacts (Zhu
463 *et al*., 2020). The possible reasons for decreasing MDA through FFM in broilers are: (1) The
464 activity of microbial proteases during the fermentation process changes substrate proteins in a
465 manner that causes to exposure of the more active R groups of amino acids. Thus, bioactive
466 peptides present in fermented products display greater antioxidant activity (Alahyaribeik *et al*.,
467 2022). Also, bioactive peptides could prevent the accumulation of cholesterol in meat
468 (Alahyaribeik *et al*., 2022), which is sensitive to oxidation. (2) Additionally, bioactive
469 polysaccharides that have antioxidant properties may be produced by fermentor microorganisms,
470 particularly fungi and bacteria (Sugiharto *et al*., 2019). (3) The live microorganisms in the
471 fermented feed including lactic acid bacteria, fungi, and *Bacillus* spp help keep the body's
472 balance of antioxidants and pro-oxidants in chickens (Sugiharto *et al*., 2019). (4) The antioxidant
473 enzymes produced during the fermentation process inhibit lipoxygenase enzymes, improve
474 oxidative stability, and increase the meat quality and shelf life (Aristides *et al*., 2018; Ibrahim *et*
475 *al*., 2021; Alahyaribeik *et al*., 2022; Xu *et al*., 2022).

476 **Immune SRBC**

477 Following the present results, previous studies have demonstrated that FFM or other fermented
478 feeds significantly increased total anti-SRBC, IgG, and IgM levels in broilers (Liu *et al*., 2020;

Xie *et al*., 2021; Xu *et al*., 2021), laying hen chicks (Zhu *et al*., 2020), and pigs (Huang *et al*., 2022). The three main immunoglobulin classes in poultry species are IgG, IgM, and IgA (Zhu *et al*., 2020), which are produced by B cells (Xu *et al*., 2021). It has been established that animal immunity and disease resistance are positively correlated with antibody contents (IgA, IgM, and IgG) (Xie *et al*., 2021). In other words, during acquired immunity, blood cells derived from B cells produce IgA, IgM, and IgG immunoglobulins in response to pathogen attack (Drażbo *et al*., 2018). The IgM is produced as the first antibody isotype in the primary antibody response (Drażbo *et al*., 2018). There are several possible mechanisms of how the FFM increased the serum concentrations of total anti-SRBC, IgG, and IgM. (1) The formation of small-sized peptides and bioactive compounds during the fermentation of feed can improve the humoral immune response of animals by stimulating B cell proliferation and the production of immunoglobulins (Zhu *et al*., 2020; Xie *et al*., 2021). (2) Additionally, the microbial fermentation process can produce a variety of advantageous compounds, including organic acids, vitamins, and exoenzymes, all of which can strengthen an animal's immune function (Gu *et al*., 2021). (3) As well as increasing lactic acid bacteria in the gut due to using of fermented protein feeds can stimulate immune cells to produce Th2 cytokines like interleukin-4 (IL-4) and IL-10, which in turn promote B cell development and antibody production (Chang *et al*., 2022). (4) The immune status of animals is highly related to antioxidant capacity. The FFM has strong antioxidant activity and this property helps to improve immune functions and reduce oxidative stress by eliminating harmful free radicals that are produced by environmental stressors or normal cellular activity (Huang *et al*., 2022).

Antibody titer against NDV

Similar outcomes to the findings of the present study have been reported in previous studies, which have indicated that fermented feed significantly increased antibody titer against NDV in broilers (Salehi *et al*., 2021; Elbaz *et al*., 2023). The generally suggested mechanisms of action in improving antibody titer against NDV by FFM may be due to the following reasons. (1) Since FFM has an antioxidant capacity, antioxidase can enhance immunity by encouraging bacterial clearance and controlling phagocyte numbers (Gu *et al*., 2021). (2) Lactic and acetic acid produced by bacteria in fermented feed results in an acidic pH 4 environment. Acidic molecules can permeate pathogen bacteria's cell membranes and increase their acidity, which disrupts enzymatic processes, kills the pathogen bacteria and modulates the immune response of poultry (Jazi *et al*., 2019). (3) Live microbes in fermented feeds can function as probiotics and stabilize intestinal inflammation by balancing the intestinal microflora, preserving the mucosal barrier, and regulating and improving the intestinal mucosal immune system response (Chang *et al*., 2022; Shihab & Nafea, 2023).

Heterophil and lymphocyte

In agreement with the current study, it has been reported that fermented feed significantly decreases the heterophil-to-lymphocyte ratio, and increases antibody titer against phytohaemagglutinin injection (Ashayerizadeh *et al*., 2017; Jazi *et al*., 2019). The heterophil-to-lymphocyte ratio is a reliable indicator for showing nutritional or environmental stress, as well as the efficiency of the body's immune response in poultry, and this ratio is increased during stress and/or illnesses (Ashayerizadeh *et al*., 2017; Jazi *et al*., 2019). Heterophils are poultry polymorphonuclear cells and are an essential part of the innate immune system (Jazi *et al*., 2019). Increasing the number of heterophils indicates the induction of innate immune responses to combat pathogenic bacteria via phagocytosis and antimicrobial activities, which include the

production of proteolytic enzymes, reactive oxygen and nitrogen species, and antimicrobial peptides (Jazi *et al*., 2019). The majority of white blood cells under normal circumstances are lymphocytes, which are produced in lymphoid tissues including the spleen, thymus, and lymph glands, and are involved in both humoral immunity and cells (Ashayerizadeh *et al*., 2017). Improvement in heterophil count and heterophil-to-lymphocyte ratio by FFM might be due to the following reasons. (1) The fermented feed has the potential to boost intestinal mucosal immunity and induce recirculating antibodies in broilers, which can lower the heterophile-to-lymphocyte ratio and reduce oxidative stress (Chang *et al*., 2022). (2) Increasing lactic acid bacteria in the gut due to using of fermented feeds stimulate mucin production and activate the immune responses to create a barrier of defense against pathogenic bacteria (Jazi *et al*., 2019). (3) Furthermore, *Bacillus subtilis* and *Bacillus licheniformis* can with bactericidal and bacteriostatic properties compete with pathogens, balance intestinal microbiota, and enhance immunity in broilers (Xu *et al*., 2021). (4) It has been shown that feeding fermented feeds to broilers increases not only antibody-mediated immune responses but also cell-mediated immune responses (Chang *et al*., 2022).

5. Conclusion

In vitro experiments of the current study demonstrated that the fermentation of FM increased its antioxidant activity, with a high performance recorded in DPPH and ABTS assays for free radical scavenging activity. In vivo experiment also showed that FFM also improved the broiler's body anti-oxidative status and contributed to the improved antioxidant enzymes, and immune

response of broilers, which resulted in increasing antibody titer against NDV, and SRBC, as well as decreasing heterophil count, and decreasing heterophil-to-lymphocyte ratio. Additionally, this study illustrated that the inclusion of FFM in broilers' diet led to an increase in the meat quality by increasing WHC, and pH, as well as reducing the lipid oxidation of meat, which reflected in the decreased MDA and increased shelf life of the product. The current study method also suggested the fermentation process through the bioreactor on an industrial scale is a sustainable method to remove the huge feather waste biomass and upgrade its feeding value to produce valuable feed ingredients from unconventional and cheaper components.

Ethical Considerations

Compliance with ethical guidelines

All animal procedures were performed following the standards outlined in the guidelines of the Animal Welfare, Ethics, Sample Collection, and Experimentation Committee of the University of Guilan (permission code: 94131/p97), Rasht, Iran.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Authors' contributions

Hassan Safari: Project administration Writing – original draft. Hassan Safari, Ardeshir Mohit, Maziar Mohiti-Asli: Conceptualization, Investigation, Methodology, Formal analysis, Data curation, Software, Supervision, Writing – review & editing.

Conflict of interest

The authors declared no conflict of interest.

Acknowledgments

This study is conducted as a part of the Ph.D. thesis of the first author. The authors would like to thank the University of Guilan for cooperation.

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پودر پَر تخمیر شده وضعیت آنتی‌اکسیدانی، کیفیت گوشت و پاسخ ایمنی جوجه‌های گوشتی را بهبود می‌بخشد

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چکیده

زمینه مطالعه: تجزیه بیولوژیکی پودر پَر یک جایگزین موثر بر سایر روش‌های فرآوری است.

هدف: این آزمایش برای بررسی اثرات پودر پَر هیدرولیز شده به روش‌های مختلف بر وضعیت آنتی‌اکسیدانی، کیفیت گوشت و پاسخ ایمنی جوجه‌های گوشتی انجام شد.

روش کار: تعداد 480 قطعه جوجه گوشتی نر یکروزه به مدت 42 روز در قالب طرح کاملاً تصادفی با هشت تیمار، پنج تکرار و 12 جوجه در هر تکرار استفاده شد. تیمارهای آزمایشی شامل: 1) گروه کنترل (جیره شاهد بدون پودر پَر)، تیمار 2، 3، 4، 5، 6، 7 و 8 به ترتیب حاوی 4 درصد پودر پَر خام، پودر پَر اتوکلاو شده، پودر پَر تخمیری با باسیلوس لیچنی‌فرمیس، پودر پُر تخمیری با باسیلوس سوبتیلیس، پودر پُر تخمیری با آسپرژیلوس نایجر، پودر پُر تخمیری با مخلوطی از باسیلوس لیچنی‌فرمیس+ باسیلوس سوبتیلیس+آسپرژیلوس نایجر و پودر پَر هیدرولیز شده با آنزیم تجاری بودند.

نتایج: مطالعه آزمایشگاهی نشان داد که فعالیت پاکسازی رادیکال‌های آزاد 2,2-دی‌فنیل-1-پیکریل‌هیدرازیل و 2,2-آزینو-بیس-3-اتیل‌بنزوتیازولین-6-سولفونیک اسید و تعداد میکروارگانیسم‌ها در پودر پَر تخمیری با افزایش زمان تخمیر افزایش یافت ($P\leq0/05$). پاکسازی رادیکال‌های آزاد 2,2-دی‌فنیل-1-پیکریل‌هیدرازیل و 2,2-آزینو-بیس-3-اتیل‌بنزوتیازولین-6-سولفونیک اسید در پودر پَر تخمیری در مقایسه با پودر پَر خام، پودر پَر اتوکلاو شده و هیدرولیز شده توسط آنزیم تجاری بیشتر بود ($P\leq0/05$). میزان pH در پودر پَر تخمیری با افزایش زمان تخمیر کاهش یافت ($P\leq0/05$). آزمایش مزرعه‌ای نشان داد که فعالیت آنزیم‌های آنتی‌اکسیدانی در جوجه‌های تغذیه شده با پودر پَر تخمیری در مقایسه با گروه کنترل و سایر تیمارهای آزمایشی بیشتر بود ($P\leq0/05$). در مقایسه با گروه کنترل و سایر تیمارهای آزمایشی، جوجه‌هایی که با پودر پَر تخمیری تغذیه شده بودند دارای سطوح مالون‌دی‌آلدهید پایین‌تری در گوشت و سرم، همچنین میزان pH و ظرفیت نگهداری آب بالاتری در روزهای 0، 7 و 14 ذخیره‌سازی گوشت نشان دادند ($P\leq0/05$). جوجه‌های تغذیه شده با پودر پَر تخمیری تیتر آنتی‌بادی بالاتری علیه ویروس نیوکاسل، تعداد کمتری هتروفیل، نسبت هتروفیل به لنفوسیت پایین‌تر، تیتر آنتی‌بادی بالاتری علیه گلبول قرمز گوسفندی (SRBC)، و همچنین ایمونوگلوبولین G و M بالاتری را در مقایسه با گروه کنترل و سایر تیمارهای آزمایشی نشان دادند ($P\leq0/05$).

نتیجه‌گیری نهایی: پودر پَر تخمیر شده وضعیت آنتی‌اکسیدانی بدن، کیفیت گوشت و پاسخ ایمنی جوجه‌های گوشتی را بهبود بخشید.

کلید واژه‌ها: پودر پر، تجزیه زیستی، تخمیر، جوجه گوشتی

785 **Table 1.** Ingredients and nutrient composition of the starter (1–10 days) diets

Ingredients (%)	Treatments[1]							
	Control	RFM	Au-HFM	Bl-FFM	Bs-FFM	An-FFM	Co-FFM	En-HFM
Corn	52.97	59.98	59.83	58.93	58.99	58.94	58.76	59.93
Soybean meal (CP: 44%)	40.12	30.58	30.71	31.58	31.56	31.6	31.8	30.65
Soybean oil	2.44	0.82	0.86	0.99	0.95	0.97	1.01	0.83
RFM	0	4	0	0	0	0	0	0
Au-HFM	0	0	4	0	0	0	0	0
Bl-FFM	0	0	0	4	0	0	0	0
Bs-FFM	0	0	0	0	4	0	0	0
An-FFM	0	0	0	0	0	4	0	0
Co-FFM	0	0	0	0	0	0	4	0
En-HFM	0	0	0	0	0	0	0	4
Dicalcium phosphate	1.9	1.89	1.88	1.86	1.86	1.86	1.86	1.88
Calcium carbonate	0.95	1	1	1	1	1	1	1
Sodium bicarbonate	0.21	0.23	0.23	0.21	0.2	0.2	0.19	0.23
Sodium chloride	0.23	0.15	0.15	0.16	0.16	0.16	0.16	0.15
Mineral and Vitamin Premix[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Met	0.34	0.32	0.31	0.3	0.3	0.3	0.3	0.31
L-Lys	0.24	0.44	0.43	0.4	0.39	0.39	0.37	0.43
L-Thr	0.11	0.1	0.1	0.08	0.08	0.08	0.05	0.1
Nutrient composition								
ME[3] (kcal/kg)	2900	2900	2900	2900	2900	2900	2900	2900
Crude protein (%)	22.23	22.23	22.23	22.23	22.23	22.23	22.23	22.23

Lys (%)	1.39	1.39	1.39	1.39	1.39	1.39	1.39	1.39
Met + Cys (%)	1.04	1.04	1.04	1.04	1.04	1.04	1.04	1.04
Thr (%)	0.94	0.94	0.94	0.94	0.94	0.94	0.94	0.94
Calcium (%)	0.93	0.93	0.93	0.93	0.93	0.93	0.93	0.93
Available phosphorus (%)	0.46	0.46	0.46	0.46	0.46	0.46	0.46	0.46
Sodium (%)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1] Control: a diet based on corn and soybean meal, without FM; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] Supplied per kg diet: vitamin A, 11 000 U; vitamin D3, 5000 U; vitamin E, 36.75 U; vitamin K3, 3.4 mg; vitamin B1,1.98 mg; vitamin B2, 5.25 mg; pantothenic acid, 10.5 mg; niacin, 31.5 mg; vitamin B6, 2.87 mg; folic acid, 1.2 mg; vitamin B12, 0.024 mg; biotin, 0.105 mg; choline, 800 mg; manganese, 120 mg; zinc, 100 mg; iron, 50 mg; copper, 12 mg; I, 1.3 mg; selenium, 0.3 mg; antioxidant, 100 mg.

[3] ME: metabolizable Energy.

795 **Table 2.** Ingredients and nutrient composition of the grower (11–24 days) diets

Ingredients (%)	**Treatments**[1]							
	Control	RFM	Au-HFM	Bl-FFM	Bs-FFM	An-FFM	Co-FFM	En-HFM
Corn	56.51	62.96	63.05	62.43	62.52	62.48	62.15	63.22
Soybean meal (CP: 44%)	35.25	26.74	26.42	26.74	26.69	26.73	27.22	26.22
Corn gluten meal	1	0.31	0.62	1	1	1	0.8	0.71
Soybean oil	3.18	1.82	1.74	1.74	1.70	1.72	1.82	1.68
RFM	0	4	0	0	0	0	0	0
Au-HFM	0	0	4	0	0	0	0	0
Bl-FFM	0	0	0	4	0	0	0	0
Bs-FFM	0	0	0	0	4	0	0	0
An-FFM	0	0	0	0	0	4	0	0
Co-FFM	0	0	0	0	0	0	4	0
En-HFM	0	0	0	0	0	0	0	4
Dicalcium phosphate	1.7	1.69	1.68	1.67	1.66	1.65	1.65	1.69
Calcium carbonate	0.88	0.91	0.92	0.92	0.93	0.93	0.93	0.92
Sodium bicarbonate	0.19	0.2	0.21	0.19	0.19	0.18	0.17	0.21
Sodium chloride	0.24	0.17	0.16	0.17	0.17	0.17	0.17	0.16
Mineral and Vitamin Premix[2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Met	0.28	0.26	0.25	0.24	0.24	0.24	0.24	0.25
L-Lys	0.2	0.37	0.38	0.35	0.36	0.36	0.33	0.38
L-Thr	0.07	0.07	0.06	0.04	0.04	0.04	0.02	0.06
Nutrient composition								
ME[3] (kcal/kg)	3000	3000	3000	3000	3000	3000	3000	3000

Crude protein (%)	20.81	20.81	20.81	20.81	20.81	20.81	20.81	20.81
Lys (%)	1.25	1.25	1.25	1.25	1.25	1.25	1.25	1.25
Met + Cys (%)	0.96	0.96	0.96	0.96	0.96	0.96	0.96	0.96
Thr (%)	0.85	0.85	0.85	0.85	0.85	0.85	0.85	0.85
Calcium (%)	0.84	0.84	0.84	0.84	0.84	0.84	0.84	0.84
Available phosphorus (%)	0.42	0.42	0.42	0.42	0.42	0.42	0.42	0.42
Sodium (%)	0.15	0.15	0.15	0.15	0.15	0.15	0.15	0.15

[1] Control: a diet based on corn and soybean meal, without FM; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus Niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] Supplied per kg diet: vitamin A, 11 000 U; vitamin D3, 5000 U; vitamin E, 36.75 U; vitamin K3, 3.4 mg; vitamin B1,1.98 mg; vitamin B2, 5.25 mg; pantothenic acid, 10.5 mg; niacin, 31.5 mg; vitamin B6, 2.87 mg; folic acid, 1.2 mg; vitamin B12, 0.024 mg; biotin, 0.105 mg; choline, 800 mg; manganese, 120 mg; zinc, 100 mg; iron, 50 mg; copper, 12 mg; I, 1.3 mg; selenium, 0.3 mg; antioxidant, 100 mg.

[3] ME: metabolizable Energy.

805 **Table 3.** Ingredients and nutrient composition of the finisher (25–42 days) diets.

Ingredients (%)	Treatments[1]							
	Control	RFM	Au-HFM	Bl-FFM	Bs-FFM	An-FFM	Co-FFM	En-HFM
Corn	60.24	67.24	67.08	66.18	66.26	66.19	66.02	67.14
Soybean meal (CP: 44%)	31.68	22.15	22.29	23.16	23.12	23.17	23.36	22.23
Soybean oil	4.34	2.72	2.76	2.89	2.85	2.88	2.91	2.75
RFM	0	4	0	0	0	0	0	0
Au-HFM	0	0	4	0	0	0	0	0
Bl-FFM	0	0	0	4	0	0	0	0
Bs-FFM	0	0	0	0	4	0	0	0
An-FFM	0	0	0	0	0	4	0	0
Co-FFM	0	0	0	0	0	0	4	0
En-HFM	0	0	0	0	0	0	0	4
Dicalcium phosphate	1.52	1.5	1.5	1.48	1.48	1.48	1.48	1.5
Calcium carbonate	0.82	0.86	0.86	0.87	0.87	0.86	0.86	0.86
Sodium bicarbonate	0.3	0.33	0.32	0.29	0.29	0.3	0.28	0.34
Sodium chloride	0.13	0.05	0.06	0.07	0.07	0.06	0.07	0.06
Mineral and Vitamin Premix [2]	0.5	0.5	0.5	0.5	0.5	0.5	0.5	0.5
DL-Met	0.26	0.23	0.23	0.22	0.22	0.22	0.21	0.23
L-Lys	0.17	0.37	0.36	0.32	0.32	0.32	0.3	0.36
L-Thr	0.05	0.04	0.04	0.02	0.02	0.02	0	0.04
Nutrient composition								
ME[3] (kcal/kg)	3100	3100	3100	3100	3100	3100	3100	3100
Crude protein (%)	18.89	18.89	18.89	18.89	18.89	18.89	18.89	18.89

Lys (%)	1.12	1.12	1.12	1.12	1.12	1.12	1.12	1.12
Met + Cys (%)	0.88	0.88	0.88	0.88	0.88	0.88	0.88	0.88
Thr (%)	0.76	0.76	0.76	0.76	0.76	0.76	0.76	0.76
Calcium (%)	0.77	0.77	0.77	0.77	0.77	0.77	0.77	0.77
Available phosphorus (%)	0.38	0.38	0.38	0.38	0.38	0.38	0.38	0.38
Sodium (%)	0.16	0.16	0.16	0.16	0.16	0.16	0.16	0.16

[1] Control: a diet based on corn and soybean meal, without FM; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] Supplied per kg diet: vitamin A, 11 000 U; vitamin D3, 5000 U; vitamin E, 36.75 U; vitamin K3, 3.4 mg; vitamin B1,1.98 mg; vitamin B2, 5.25 mg; pantothenic acid, 10.5 mg; niacin, 31.5 mg; vitamin B6, 2.87 mg; folic acid, 1.2 mg; vitamin B12, 0.024 mg; biotin, 0.105 mg; choline, 800 mg; manganese, 120 mg; zinc, 100 mg; iron, 50 mg; copper, 12 mg; I, 1.3 mg; selenium, 0.3 mg; antioxidant, 100 mg.

[3] ME: metabolizable energy.

Table 4. Antioxidant potential and physiochemical characteristics of various fermented feather meals at different times of fermentation.

Treatments[1]	Fermentation times (days)				SEM	*P-Value*
	0[4]	4	8	12		
Antioxidant potential						
Bl-FFM						
DPPH[2] scavenging, (%)	3.17 [d]	13.20 [c]	24.70 [b]	39.44 [a]	2.194	0.001
ABTS[3] scavenging, (%)	4.28 [d]	15.24 [c]	29.24 [b]	47.35 [a]	2.604	0.001
Bs-FFM						
DPPH scavenging, (%)	3.17 [d]	13.52 [c]	24.81 [b]	38.58 [a]	2.137	0.001
ABTS scavenging, (%)	4.28 [d]	14.72 [c]	27.73 [b]	46.14 [a]	2.526	0.001
An-FFM						
DPPH scavenging, (%)	3.17 [d]	12.87 [c]	23.87 [b]	37.92 [a]	2.104	0.001
ABTS scavenging, (%)	4.28 [d]	14.92 [c]	28.38 [b]	45.96 [a]	2.515	0.001
Co-FFM						
DPPH scavenging, (%)	3.17 [d]	14.13 [c]	25.11 [b]	40.11 [a]	2.214	0.001
ABTS scavenging, (%)	4.28 [d]	15.63 [c]	30.36 [b]	49.04 [a]	2.704	0.001
pH value						
Bl-FFM	7.00 [a]	6.68 [a]	5.63 [b]	4.45 [c]	0.168	0.001
Bs-FFM	6.98 [a]	6.73 [a]	5.71 [b]	4.36 [c]	0.174	0.001
An-FFM	7.02 [a]	6.80 [a]	5.61 [b]	4.38 [c]	0.177	0.001
Co-FFM	6.98 [a]	6.51 [b]	5.55 [c]	4.25 [d]	0.175	0.001
Microorganisms count, log CFU/g feed						
Bl-FFM	6.73 [d]	7.94 [c]	8.97 [b]	9.83 [a]	0.185	0.0001
Bs-FFM	6.71 [d]	7.96 [c]	8.95 [b]	9.84 [a]	0.187	0.0001
An-FFM	6.72 [d]	7.95 [c]	8.99 [b]	9.87 [a]	0.188	0.0001
Co-FFM	6.70 [d]	7.94 [c]	8.98 [b]	9.85 [a]	0.188	0.0001

[a-d] Means within a row with different superscripts differ significantly ($P \leq 0.05$).

[1]Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis*+ *Bacillus subtilis*+ *Aspergillus niger*. [2]DPPH: 2,2-diphenyl-1-picrylhydrazl; [3]ABTS: 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid; [4]0-day: initial condition.

822 **Table 5.** Antioxidant potential of various hydrolyzed feather meals (The 12th day of fermentation in fermented
823 feather meals).

	Parameters	
Treatments[1]	DPPH[2] free radical scavenging activity, (%)	ABTS[3] free radical scavenging activity, (%)
RFM	3.17 [b]	4.28 [b]
Au-HFM	4.23 [b]	5.15 [b]
Bl-FFM	39.44 [a]	47.35 [a]
Bs-FFM	38.58 [a]	46.14 [a]
An-FFM	37.92 [a]	45.96 [a]
Co-FFM	40.11 [a]	49.04 [a]
En-HFM	5.07 [b]	6.11 [b]
SEM	2.1	2.514
P-value	0.001	0.001

824 [a-c] Means within a column with different superscripts differ significantly ($P \leq 0.05$).
825 [1] RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by
826 *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by
827 *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis*+ *Bacillus subtilis*+ *Aspergillus niger*;
828 En-HFM: hydrolyzed feather meal by an enzyme.
829 [2] DPPH: 2,2-diphenyl-1-picrylhydrazl; [3]ABTS: 2, 2′-azino-bis-3-ethylbenzthiazoline-6-sulfonic acid.

Table 6. Effects of various hydrolyzed feather meals on antioxidant enzyme activities in serum of broilers at 42 d of age.

Treatments[1]	Antioxidant enzyme activities[2] (U/mL)			
	TAC	GPX	SOD	CAT
Control	9.93 [b]	210.42 [d]	124.93 [d]	4.12 [b]
RFM	10.23 [b]	215.22 [cd]	128.06 [cd]	4.22 [b]
Au-HFM	10.41 [b]	223.54 [bc]	133.41 [bc]	4.25 [b]
Bl-FFM	13.82 [a]	285.66 [a]	173.48 [a]	6.88 [a]
Bs-FFM	13.70 [a]	288.30 [a]	173.64 [a]	6.92 [a]
An-FFM	13.61 [a]	279.40 [a]	170.08 [a]	6.71 [a]
Co-FFM	14.01 [a]	290.26 [a]	176.24 [a]	7.02 [a]
En-HFM	10.72 [b]	230.65 [b]	139.08 [b]	4.30 [b]
SEM	0.226	3.274	2.100	0.126
P-Value	0.001	0.001	0.001	0.001

[a-c] Means within a column with different superscripts differ significantly ($P \leq 0.05$).

[1] Control: diet based on corn and soybean meal, without feather meal; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] TAC: total antioxidant capacity; GPX: glutathione peroxidase; SOD: superoxide dismutase; CAT: catalase.

Table 7. Effects of various hydrolyzed feather meals on malondialdehyde concentrations in serum at 42 d of age and breast meat quality of broilers during 14 d of storage.

Treatments[1]	Breast meat									Serum
	MDA[2], (nmol/mg of protein)			pH			WHC[3], (%)			
	0 d	7 d	14 d	0 d	7 d	14 d	0 d	7 d	14 d	MDA, (nmol/mL)
Control	0.456 [a]	0.764 [a]	1.211 [a]	6.11 [b]	5.86 [b]	5.53 [b]	82.15 [b]	75.46 [b]	65.46 [b]	6.12 [a]
RFM	0.451 [ab]	0.762 [a]	1.196 [a]	6.12 [b]	5.88 [b]	5.56 [b]	82.18 [b]	76.12 [b]	66.49 [b]	6.10 [a]
Au-HFM	0.445 [ab]	0.753 [a]	1.178 [a]	6.15 [b]	5.89 [b]	5.56 [b]	83.17 [b]	76.42 [b]	68.27 [b]	5.98 [a]
Bl-FFM	0.280 [c]	0.442 [b]	0.668 [b]	6.84 [a]	6.60 [a]	6.32 [a]	94.12 [a]	89.17 [a]	86.19 [a]	2.88 [b]
Bs-FFM	0.278 [c]	0.446 [b]	0.664 [b]	6.85 [a]	6.61 [a]	6.35 [a]	94.02 [a]	90.41 [a]	85.66 [a]	2.83 [b]
An-FFM	0.283 [c]	0.452 [b]	0.678 [b]	6.81 [a]	6.58 [a]	6.29 [a]	93.04 [a]	89.04 [a]	85.73 [a]	2.94 [b]
Co-FFM	0.275 [c]	0.439 [b]	0.653 [b]	6.87 [a]	6.64 [a]	6.35 [a]	95.10 [a]	91.11 [a]	88.55 [a]	2.80 [b]
En-HFM	0.441 [b]	0.745 [a]	1.171 [a]	6.17 [b]	5.92 [b]	5.60 [b]	85.58 [b]	78.20 [b]	70.73 [b]	5.98 [a]
SEM	0.007	0.014	0.024	0.033	0.034	0.036	0.697	0.790	1.073	0.146
P-Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001

[a-c] Means within a column with different superscripts differ significantly ($P \leq 0.05$).

[1] Control: diet based on corn and soybean meal, without feather meal; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] MDA: malondialdehyde; [3] WHC: water-holding capacity.

Table 8: Effects of various hydrolyzed feather meals on antibody response against sheep red blood cell (SRBC, log_2), Newcastle disease virus (NDV, log_2), heterophil, and lymphocyte in broilers.

Treatments[1]	SRBC[2] titer at 35 d of age			SRBC titer at 42 d of age			Antibody titer against NDV		leucocytes count 42 d of age[5]		
	Total anti-SRBC	IgG[3]	IgM[4]	Total anti-SRBC	IgG	IgM	24 d	42 d	H, (%)	L, (%)	H:L ratio
Control	3.13 ^{b}	1.53 ^{b}	1.60 ^{b}	4.33 ^{c}	2.20 ^{b}	2.13 ^{b}	5.00 ^{b}	3.13 ^{b}	33.20 ^{a}	51.33	0.654 ^{a}
RFM	3.33 ^{b}	1.66 ^{b}	1.66 ^{b}	4.66 ^{bc}	2.33 ^{b}	2.33 ^{b}	5.13 ^{b}	3.26 ^{b}	36.13 ^{a}	52.26	0.696 ^{a}
Au-HFM	3.4 ^{b}	1.66 ^{b}	1.73 ^{b}	4.66 ^{bc}	2.46 ^{b}	2.20 ^{b}	5.13 ^{b}	3.33 ^{b}	34.33 ^{a}	52.40	0.664 ^{a}
Bl-FFM	7.33 ^{a}	4.13 ^{a}	3.20 ^{a}	9.33 ^{a}	5.33 ^{a}	4.00 ^{a}	8.33 ^{a}	5.53 ^{a}	19.13 ^{b}	55.13	0.353 ^{b}
Bs-FFM	7.46 ^{a}	4.13 ^{a}	3.33 ^{a}	9.33 ^{a}	5.13 ^{a}	4.20 ^{a}	8.26 ^{a}	5.46 ^{a}	19.20 ^{b}	54.33	0.357 ^{b}
An-FFM	7.06 ^{a}	4.26 ^{a}	2.80 ^{a}	9.00 ^{a}	5.06 ^{a}	3.93 ^{a}	8.13 ^{a}	5.33 ^{a}	20.06 ^{b}	54.06	0.374 ^{b}
Co-FFM	7.60 ^{a}	4.40 ^{a}	3.20 ^{a}	9.60 ^{a}	5.33 ^{a}	4.26 ^{a}	8.46 ^{a}	5.60 ^{a}	18.20 ^{b}	55.13	0.336 ^{b}
En-HFM	3.4 ^{b}	1.66 ^{b}	1.73 ^{b}	5.00 ^{b}	2.66 ^{b}	2.33 ^{b}	5.20 ^{b}	3.33 ^{b}	34.26 ^{a}	52.33	0.662 ^{a}
SEM	0.193	0.126	0.084	0.220	0.135	0.105	0.157	0.115	0.736	0.556	0.015
P-Value	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.001	0.307	0.001

[a-c] Means within a column with different superscripts differ significantly ($P \leq 0.05$).

[1] Control: diet based on corn and soybean meal, without feather meal; RFM: raw feather meal; Au-HFM: hydrolyzed feather meal by autoclave; Bl-FFM: fermented feather meal by *Bacillus licheniformis*; Bs-FFM: fermented feather meal by *Bacillus subtilis*; An-FFM: fermented feather meal by *Aspergillus niger*; Co-FFM: fermented feather meal by *Bacillus licheniformis* + *Bacillus subtilis* + *Aspergillus niger*; En-HFM: hydrolyzed feather meal by an enzyme.

[2] SRBC, sheep red blood cell; [3] IgG: immunoglobulin G; [4] IgM, immunoglobulin M.

[5] H: heterophile; L: lymphocyte; H/L ratio: heterophile to lymphocyte ratio