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Improving Broiler Chicken Intestinal Microbiota and Immune Function Using Black Cumin Seed Bioactive Peptides: A Comparative Study with Prebiotics and Organic Acids

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Abstract

This research investigated the impact of hydrolyzed black cumin seed protein (HBCP) as a potential source of bioactive peptides on the performance of broiler chickens. A total of 560 Ross 308 broiler chicks were randomly assigned to seven distinct dietary groups: Control, 0.05% HBCP, 0.1% HBCP, 0.15% HBCP, 0.2% HBCP, 0.2% prebiotic and 0.2% organic acid. The experiment was conducted over a 42-day period. Birds were fasted for 8 hours before weighing, and feed intake was recorded daily to calculate the feed conversion ratio (FCR). For microbial and enzyme analyses, two birds per replicate were selected and euthanized, with intestinal samples collected under sterile conditions. Serum samples were taken on day 42 to assess liver enzyme concentrations, and immune response was measured using a sheep red blood cell assay. The results indicated that including 0.1%, 0.15%, and 0.2% HBCP in the broiler diets significantly improved body weight gain and feed conversion ratio throughout the rearing period. Additionally, the inclusion of 0.2% prebiotic and organic acid in the diet notably increased body weight and decreased feed conversion ratio compared to the control group (P<0.05). These dietary interventions had a positive effect on the intestinal microbiota, decreasing *Escherichia coli* counts while boosting *Lactobacillus* populations (P<0.05). Moreover, supplementation with HBCP, prebiotics, and organic acids improved immune function, as indicated by elevated antibody titers and improved liver enzyme profiles (P<0.05). In conclusion, bioactive peptides derived from black cumin hydrolysate exhibited effects similar to prebiotics and organic acids. These findings highlight hydrolyzed black cumin protein as a promising natural alternative to conventional prebiotics and organic acids in broiler nutrition.

Introduction

Antimicrobials have been widely utilized as sub-therapeutic growth promoters in poultry diets due to their beneficial effects on growth performance and health (Paul *et al*., 2022). However, the widespread use of antimicrobials has significantly contributed to the development of antimicrobial resistance, highlighting the need for sustainable alternatives (Gadde *et al*., 2017). Probiotics, prebiotics, essential oils, natural products, and bioactive peptides have emerged as promising alternatives to antimicrobials in poultry nutrition. These additives can enhance poultry performance by reducing the proliferation of pathogenic bacteria in the gastrointestinal tract and

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improving nutrient utilization (Ağagündüz *et al*., 2023).

For instance, probiotics promote the growth of beneficial bacteria and maintain gut barrier integrity, while prebiotics support the growth of healthy microbes in the gut (Surolia *et al*., 2024). Essential oils and natural products, such as plant extracts, have shown antimicrobial, antiviral, antifungal, and antioxidant properties, contributing to improved animal performance and health (Ağagündüz *et al*., 2023). Although antimicrobials have shown positive effects on poultry performance, their excessive use has contributed to the emergence of cross-resistance and multidrug resistance in pathogenic bacteria. This has made it essential to explore sustainable and environmentally safe alternatives to tackle the increasing challenge of antimicrobial resistance.

Black cumin (*Nigella sativa*), an aromatic plant native to Asia and the Mediterranean region, is a valuable source of nutrients. It contains approximately 21.07% protein, 5.02% moisture, 0.5-1.5% essential oil, and various amino acids, including arginine, glutamic acid, and leucine (Hannan *et al*., 2021). The by-product of oil extraction from black cumin seeds, referred to as black cumin seed cake, is also rich in nutrients (Hossain *et al*., 2024). The residue remaining after oil extraction from black cumin seeds, known as black cumin seed cake, is also nutrient-dense. Black cumin seed cake is nutrient-dense, containing high levels of crude protein (29.84%), oil (4.31%), and 1949 kcal of apparent metabolizable energy (AME) for poultry, and is also an excellent source of fiber, carbohydrates, vitamins, phenols, and antioxidants (Albakry *et al*., 2022; Hannan *et al.,* 2021). To fully utilize the nutritional benefits of black cumin, it is essential to employ innovative processing methods such as protein hydrolysis, which breaks down proteins into peptides—short chains of amino acids that serve various biological functions (Cruz-Casas *et al*., 2021; Nasiri *et al*., 2022).

Intestinal cells can readily absorb these peptides, which exhibit various therapeutic properties. For instance, they demonstrate antioxidant effects (Gao *et al.,* 2010; Power *et al.,* 2013), antimicrobial activity (Osman *et al*., 2016; Wald *et al*., 2016; Ryder *et al*., 2016; Kotzamanis *et al*., 2007), and immune-modulating capabilities (Zambrowicz *et al*., 2015; Hisham *et al*., 2018). Additionally, some peptides have shown antihypertensive potential (Hisham *et al*., 2018). This study aimed to evaluate the potential of hydrolyzed black cumin seed cake protein (HBCP) as a valuable source of bioactive peptides for broiler chicken diets, comparing its effects to those of prebiotics and organic acids.

Materials and Methods

Preparation of Bioactive Peptides from Black Cumin Seed Cake

The peptide extraction process from black cumin seed cake was adapted from Karimzadeh *et al*. (2017) with slight modifications. Initially, black cumin seed cake powder was combined with distilled water, and the pH was set to 10. The mixture was then heated to 45°C, cooled down to room temperature and centrifuged at 11,000 × g for 30 minutes. Afterwards, the pH of the supernatant was adjusted to 4.5 using a 1 M hydrochloric acid solution, and another round of centrifugation followed. The resulting protein precipitate was then dissolved in distilled water, and the pH was adjusted to 7. Finally, the solution was frozen at -30°C and lyophilized to obtain black cumin seed cake protein isolate.

To produce hydrolyzed protein from black cumin seed cake, the protein isolate was dissolved at a 5% concentration in a 250 mL bioreactor. The temperature and pH of the solution were adjusted to optimal conditions for enzyme activity before starting the hydrolysis. The hydrolysis vessel was placed on a magnetic stirrer hotplate, and the mixture was continuously stirred throughout the process. Hydrolysis was performed using a protease enzyme (Novozymes, Denmark) at 50°C and pH 8 for 4 hours, with an enzyme-to-protein ratio of 1:20. During hydrolysis, the pH was maintained at 8 using 1 M sodium hydroxide. After hydrolysis, the pH was adjusted to 4 using 1 M hydrochloric acid, and the mixture was heated in boiling water for 10 minutes to inactivate the enzyme. The mixture was then centrifuged at 8000 rpm for 30 minutes to remove impurities. The supernatant was poured into large 15 cm diameter Petri dishes and frozen at -30°C. Finally, the frozen solution was lyophilized to obtain black cumin seed cake peptide powder (Karimzadeh *et al*., 2017).

Characterization of Bioactive Peptides from Black Cumin Seed Cake

To analyze the molecular weight distribution and concentration of bioactive peptides in black cumin seed cake protein, an HPLC system was utilized. The setup included a TSK 3000 gel PWXL column (A specific type of gel filtration chromatography column) and a mobile phase composed of a 1:1 (v/v) mixture of acetonitrile and water, with 0.1% trifluoroacetic acid (TFA). The eluted peptides' absorbance was measured at 225 nm, with a flow rate of 0.5 mL/min. Molecular weight standards such as bovine serum albumin (BSA), cytochrome C, bacitracin, and reduced glutathione were used to calibrate the system. The concentration of the bioactive peptide protein in lyophilized black cumin seed cake protein was quantified using the Biuret method, with BSA as a reference. The Biuret test

relies on the formation of a purple-colored complex between Cu (II) ions and peptide bonds under alkaline conditions.

Experimental Design and Animal Management

The ethics committee of the University of Mohaghegh Ardabili Iran, approved this study. A total of 560 day-old Ross 308 broiler chicks were randomly distributed into seven different dietary groups in a fully randomized design, with five replicates consisting of 16 birds each. The experimental diets were formulated to meet the nutritional requirements of the Ross 308 strain as recommended by Aviagen (2019) for the starter (1-10 days), grower (11-24 days), and finisher (25-42 days) phases (Table 1). Seven dietary treatments were tested. These included a control (no additive), and diets containing 0.05%, 0.1%, 0.15%, or 0.2% HBCP, 0.2% prebiotic (TechnoMos, Biochem), or 0.2% organic acid (Flora Gold, Sepahan Daneh, Iran).

Environmental conditions, including temperature, humidity, and lighting, were maintained according to the recommendations of the Ross 308 strain catalog. Vaccination was carried out according to the local veterinary department's schedule. Birds had ad libitum access to water and feed throughout the experiment. The experimental diets were formulated using the UFFDA diet formulation software, incorporating the National Research Council (NRC) tables for feedstuffs and the requirements outlined in the Ross 308 commercial strain rearing guide (Table 1).

Table 1: Basal diets and their chemical compositions for the starter (0-10 days), grower (11-24 days), and finisher (25-42 days) phases

Ingredients (%)	Starter	Grower	Finisher
Corn	50.59	52.09	53.36
Soybean Meal	40.81	37.87	36.65
Vegetable oil	3.81	5.45	5.88
Dicalcium phosphate	1.89	1.92	1.64
Seashell powder	1.33	1.21	1.11
Salt	0.42	0.42	0.42
DL-methionine	0.36	0.28	0.21
L-lysine hydrochloride	0.29	0.26	0.23
Vitamin supplement*	0.25	0.25	0.25
Mineral supplement **	0.25	0.25	0.25
Calculated chemical composition			
Metabolizable energy (kcal/kg)	2975	3100	3150
Protein (%)	22.62	21.50	21
Lysine (%)	1.450	1.360	1.305
Methionine (%)	0.701	0.606	0.532
Methionine + Cystine (%)	1.060	0.950	0.870
Arginine (%)	1.464	1.387	1.353
Calcium (%)	1.05	1.0	0.90
Available phosphorus (%)	0.5	0.5	0.445
Sodium (%)	0.18	0.18	0.18

*****Provided per kilogram of diet: Vitamins: Vitamin A (18,000 IU), vitamin E (72 mg), vitamin K3 (4 mg), vitamin B1 (3.55 mg), vitamin B2 (1.13 mg), calcium pantothenate (19.06 mg), niacin (59.04 mg), vitamin B6 (88.5 mg), vitamin B9 (2 mg), vitamin B12 (0.03 mg), and choline chloride (1 g).

******Provided per kilogram of diet: Manganese (198.04 mg), zinc (169.04 mg), iron (100 mg), copper (20 mg), iodine (1.985 mg), and selenium (0.04 mg).

Data Collection

To evaluate growth performance during the rearing period, we measured daily weight gain and feed intake while also accounting for mortality rates. Before weighing, the birds were fasted for approximately 8 hours. The feed conversion ratio was calculated by dividing the total feed intake of each experimental unit by the corresponding weight gain.

Intestinal Microbial Population and Digestive Enzymes

At 42 days of age, two birds per replicate, with weights close to the experimental unit average, were selected and euthanized to evaluate digestive enzyme activities and determine the intestinal microbial population. The terminal ileum, from Meckel's diverticulum to the cecum and colon junction, was carefully excised with sterile scissors and both ends tightly ligated with sterile thread. The samples were then placed in sterile containers at 4°C and transported to the laboratory for further analysis. One unit of amylase activity was defined as the amount of enzyme that hydrolyzes one milligram of glucose, using corn starch as the substrate (Hagberg, 1960).

One unit of lipase activity was defined as the volume (in mL) of 0.05 M sodium hydroxide required to neutralize the free fatty acids released from 3 mL of olive oil substrate after a 6-hour incubation at 38°C

(Tietz and Fiereck, 1966). Similarly, a unit of protease activity was defined as the enzyme quantity required to hydrolyze 1 mg of azocasein (Kunitate *et al*., 1989). To determine total bacterial counts, tryptic soy agar was utilized. After preparing the culture medium, 0.1 mL of the sample was spread on the surface using a micropipette. If necessary, samples with high bacterial counts were diluted up to six logs in physiological saline. The culture plates were incubated at 35°C for 48 hours before counting the colonies (Kunitate *et al.,* 1989). For Bacillus bacteria counts, nutrient agar was used. A 0.1 mL aliquot of the diluted intestinal sample was spread on the culture medium surface. The samples were incubated aerobically at 37°C for 48 hours. After incubation, the colonies were counted, multiplied by the dilution factor, and the logarithm was calculated to obtain the log of colony-forming units per gram (log cfu/g).

Serum and Liver Enzyme Concentrations

The blood samples were collected from birds that were distinct from those used for intestinal microbial population and digestive enzyme analyses. To ensure consistency and avoid excessive handling stress, separate birds were designated for each type of sample collection. Blood was obtained via venipuncture from the brachial vein, using sterile syringes and anticoagulant-treated tubes. Samples were collected on day 42 from the wing vein and immediately processed for serum separation. This method ensured the integrity of the blood samples for subsequent analyses of liver enzymes and immune response markers.

Serum samples collected on day 42 of the rearing period were analyzed to determine liver enzyme concentrations. Alanine aminotransferase (ALT) and aspartate aminotransferase (AST) levels were measured using specific kits (PARS Peyvand, Iran), and an autoanalyzer (Eppendorf 5060) at a wavelength of 340 nm and a temperature of 37°C, in accordance with the guidelines of the International Federation of Clinical Chemistry (IFCC, 2018).

Immune Response

At 14 days of age, two birds from each replicate were intravenously injected with 0.1 mL of a 0.5% diluted sheep red blood cell (SRBC) solution. Seven days later (at 21 days of age), blood samples were collected from the same birds. After allowing the blood to coagulate for 16 hours, the serum was separated. The antibody titer against SRBC was determined using a microtiter hemagglutination assay (Isakov *et al*., 2005). To measure the titer of 2-mercaptoethanol-resistant antibodies (IgG), 50 micromolar of 2-mercaptoethanol was used. The immunoglobulin M (IgM) titer was calculated by subtracting the titer of 2-mercaptoethanol-resistant antibodies (IgG) from the total SRBC response titer (Giorgio *et al*., 2002).

Statistical Analysis

Analysis of the collected data was performed using the General Linear Model (GLM) procedure in SAS 9.4 (2016). Duncan's multiple range test was then applied to compare means, with a significance level of 0.05.

Results

Bioactive Peptides

The molecular weight distribution of bioactive peptides in black seed protein reveals a significant variation across different ranges (Table 2). The majority of bioactive peptides fall within the 180-500 Da range, accounting for 55.87% of the total. This is followed by peptides in the 500-1000 Da range, which make up 27.66%. Peptides with molecular weights between 1000-2000 Da constitute 10.60%, while those in the 2000-3000 Da range represent 0.89%. The smallest fractions are found in the <180 Da and >3000 Da ranges, comprising 4.80% and 0.09%, respectively. This distribution highlights the predominance of lower molecular weight peptides in black seed protein.

Table 2: Molecular weight distribution of bioactive peptides in black seed protein

Molecular weight range (Da)	Bioactive peptides %
>3000	0.09
2000-3000	0.89
1000-2000	10.60
500-1000	27.66
180-500	55.87
<180	4.80

Growth Performance

Table 3 summarizes the impact of various experimental treatments on the growth performance of broilers during the experimental period (days 1-42). The results indicate that dietary supplementation with HBCP (Hydrolyzed black cumin seed protein) significantly influenced broiler performance. Broilers receiving 0.2% HBCP showed the highest daily weight gain at 64.59 g/b/d and the lowest FCR of 1.60, significantly surpassing the control group, which had a weight gain of 59.63 g/b/d and an FCR of 1.79 ($P< 0.001$). Additionally, treatments with 0.1%, 0.15%, and 0.2% HBCP, as well as prebiotic and organic acid supplements, resulted in significantly improved feed conversion ratios compared to the control ($P< 0.0001$). These findings suggest that HBCP, particularly at higher inclusion levels, can enhance growth performance and feed efficiency in broilers.

Table 3: Effects of different experimental treatments on broiler growth performance during the experimental period (1-42 days)

Treatments	Daily Feed Intake g/b/d)	Daily Weight Gain (g/b/d)	Feed Conversion Ratio
Control	107.1	59.60^{c}	1.79^{a}
0.05% HBCP	106.2	60.20^{bc}	1.76^{a}
0.1% HBCP	105.0	62.0^{bc}	1.69^{b}
0.15% HBCP	104.4	63.4^{ab}	1.64^{c}
0.2% HBCP	103.6	64.6^{a}	1.60^{c}
Prebiotic	104.0	63.9^{ab}	1.63^{c}
Organic Acid	104.4	63.4^{ab}	1.65^{c}
SEM	1.81	0.78	0.014
P Value	0.800	0.0004	0.0001

HBCP: Hydrolyzed Black Cumin Seed Protein.
$^{a-e}$ Different letters in columns indicate a significant difference at the 5% level.

Microbial Population

The experimental treatments had a significant impact on the ileal bacterial population in broilers throughout the growing period (Table 4). *Lactobacillus* counts were highest in the groups receiving 0.2% HBCP, prebiotics, and organic acids, with significant increases observed compared to the control group ($P<$ 0.0001). In contrast, *Escherichia coli* counts were significantly lower in these groups, with the lowest levels found in the 0.2% HBCP group ($P<$ 0.0001). Total bacterial counts displayed a similar pattern, being highest in the 0.2% HBCP, prebiotic, and organic acid groups, which were significantly greater than those in the control group ($P<$ 0.0001). These results indicate that higher levels of HBCP, prebiotics, and organic acids can beneficially modulate the ileal bacterial population in broilers.

Table 4: Effects of experimental treatments on ileal bacterial populations in broiler chickens (log cfu/g)

Treatments	Total bacteria	*Escherichia coli*	*Lactobacillus*
Control	5.1^{c}	3.37^{a}	4.81^{c}
0.05% HBCP	5.06^{c}	3.36^{a}	4.82c
0.1% HBCP	5.47^{b}	2.36^{b}	5.17^{b}
0.15% HBCP	5.48^{b}	2.35^{b}	5.20^{b}
0.2% HBCP	6.13^{a}	1.90^{c}	5.89^{a}
Prebiotic	6.14^{a}	1.89^{c}	5.85^{a}
Organic Acid	6.13^{a}	1.91^{c}	5.82^{a}
SEM	0.115	0.013	0.043
P Value	0.0001	0.0001	0.0001

HBCP: Hydrolyzed black cumin seed protein.
$^{a-c}$ Different letters in columns indicate a significant difference at the 5% level.

Immune System and Liver Enzymes

Table 5 shows that, significant effects of the experimental treatments on the immune system and liver enzymes in broilers. SRBC titers were highest in the prebiotic group (8.7) and lowest in the control group (6.2) ($P<$ 0.0001). IgG levels were significantly elevated in the 0.15% HBCP, 0.2% HBCP, prebiotic, and organic acid groups compared to the control ($P<$ 0.0001). IgM levels were highest in the 0.2% HBCP, prebiotic, and organic acid groups ($P<$ 0.05). AST levels were significantly reduced in the 0.2% HBCP group (9.6) compared to the control (13.0) ($P<$ 0.0001). ALT levels were lowest in the 0.2% HBCP group (218.0) and highest in the control group (264.8) ($P<$ 0.0001). These findings suggest that HBCP, prebiotics, and organic acids positively influence immune responses and liver enzyme activities in broilers.

Table 5: Effect of experimental treatments on the immune system and liver enzymes in broilers.

Treatments	SRBC titer (Log2)	IgG (U/mL)	IgM (U/mL)	AST (U/L)	ALT (U/L)
Control	6.2^{d}	3.3^{c}	2.9^{b}	13.0^{a}	264.8^{a}
0.05% HBCP	6.3^{d}	3.2^{c}	3.1^{b}	12.4^{ab}	260.0^{a}
0.1% HBCP	7.2^{c}	4.0^{b}	3.3^{ab}	11.2^{bc}	239.8^{bc}
0.15% HBCP	8.1^{b}	4.6^{a}	3.5^{ab}	10.4^{cd}	230.4^{d}
0.2% HBCP	8.6^{ab}	4.8^{a}	3.8^{a}	9.6^{d}	218.0^{e}
Prebiotic	8.7^{a}	4.7^{a}	3.8^{a}	10.0^{cd}	232.2^{cd}
Organic Acid	8.4^{ab}	4.6^{a}	3.8^{a}	12.4^{ab}	244.4^{b}
SEM	0.185	0.16	0.209	0.47	2.74
P Value	0.0001	0.0001	0.017	0.0001	0.0001

HBCP: Hydrolyzed black cumin seed protein.
$^{a-c}$ Different letters in columns indicate a significant difference at the 5% level.

Digestive Enzymes

The results of the study, as shown in Table 6, indicate that the experimental treatments had a significant impact on the digestive enzyme activities in broilers. The control group exhibited the lowest enzyme activities, with protease at 78.74 U/mL, lipase at 19.22 U/mL, and amylase at 8.42 U/mL ($P < 0.0001$). The addition of 0.05% HBCP did not significantly alter these values. However, increasing the HBCP concentration to 0.1% and 0.15% significantly enhanced enzyme activities ($P < 0.0001$). The highest enzyme activities were observed in the 0.2% HBCP, prebiotic, and organic acid groups, with protease around 90.82-90.84 U/mL, lipase around 23.65-23.68 U/mL, and amylase around 10.52-10.53 U/mL ($P < 0.0001$). The findings indicate that digestive enzyme activities in broilers are significantly improved by higher concentrations of HBCP, as well as by prebiotics and organic acids.

Table 6: Effect of experimental treatments on digestive enzymes in broilers (U/mL)

Treatments	Amylase	Lipase	Protease
Control	8.42[c]	19.22[c]	78.74[c]
0.05% HBCP	8.44[c]	19.21[c]	78.73[c]
0.1% HBCP	9.37[b]	20.56[b]	82.13[b]
0.15% HBCP	9.38[b]	20.55[b]	82.11[b]
0.2% HBCP	10.53[a]	23.68[a]	90.82[a]
Prebiotic	10.52[a]	23.66[a]	90.84[a]
Organic Acid	10.53[a]	23.65[a]	90.83[a]
SEM	0.017	0.02	0.025
P Value	0.0001	0.0001	0.0001

HBCP: Hydrolyzed black cumin seed protein.
[a-c] Different letters in columns indicate a significant difference at the 5% level.

Discussion

The protein hydrolysate of black cumin seed protein mainly contains peptides in the molecular weight range of 180-500 Da, with smaller amounts in the 500-1000 Da and 1000-2000 Da ranges. Their molecular weight influences the bioactivity of peptides, but there is no universal range that determines bioactivity. Both short peptides (oligopeptides) and larger peptides can exhibit significant biological effects, depending on the specific context and target (Wang *et al*., 2016). The present study revealed that increasing levels of bioactive peptides extracted from black cumin seed cake significantly reduced the feed conversion ratio in broiler chickens throughout the entire rearing period, without influencing feed intake. Despite numerous reports on the effects of black seed or its derivatives on broilers (Saeid *et al*., 2013), there is no report on the effects of black seed bioactive peptides. Therefore, several reports on the effects of bioactive peptides extracted from other plant sources are mentioned.

A study by Abdollahi *et al*. (2018) assessing soybean bioactive peptides (SBP) in broilers found that SBP supplementation significantly improved the feed conversion ratio (FCR). Specifically, inclusion levels of 5.0 and 6.0 g/kg resulted in lower (better) FCR compared to diets lacking SBP. While the same study noted no significant impact on weight gain or feed intake, it did observe trends towards enhanced nutrient digestibility and improved intestinal histology, including increased villus height in birds fed higher SBP levels. Research on bioactive peptides derived from sesame meal showed improvements in growth performance, gut microbiota composition, and intestinal morphology in broilers. Higher inclusion levels of sesame meal peptides enhanced overall health and performance (Sa'adoon and Abbas, 2023).

Studies on cottonseed bioactive peptides indicated that their supplementation improved productive performance and FCR in broilers, highlighting their potential as a functional protein source in poultry diets (Landy *et al*., 2020). Mohammadrezaei *et al*. (2021) evaluated the effects of cottonseed meal bioactive peptides (CSBP) on broiler performance, finding that birds receiving 20 g/kg CSBP had increased feed intake and improved FCR over 1–35 days compared to the control group. Broilers that were fed a supplement of 15 g/kg CSBP showed significant improvements in livability, the European Production Efficiency Factor (EPEF), and the European Broiler Index (EBI).

Bioactive peptides enhance broiler performance through several mechanisms. They improve nutrient absorption by enhancing gut morphology and enzyme activity, leading to better feed efficiency and growth rates (Salavati *et al*., 2020). Their antimicrobial properties help maintain a healthy gut microbiota, reducing pathogenic bacteria and promoting overall gut health (Salavati *et al*., 2021). Additionally, bioactive peptides boost the immune system by increasing antibody production and immune cell activity, aiding broilers in resisting infections (Wei *et al*., 2024). Their antioxidant properties reduce oxidative stress, improving cellular function and overall health (Abdollahi *et al*., 2017). These combined effects contribute to improved growth

performance, feed efficiency, and health in broilers (Urban *et al*., 2024).

In the present study, a reduction in *Escherichia coli* and an increase in *Lactobacillus* bacteria occurred when levels of peptides from black cumin seed cake were increased in broiler diets. Similarly, adding prebiotics and organic acids to the diet also decreased E. coli and increased *Lactobacillus*. Various studies have highlighted the positive effects of bioactive peptides on the intestinal microbiota of broilers. For instance, Seifi *et al*. (2018) and Karimzadeh *et al*. (2016) observed reductions in pathogenic bacteria like E. coli and coliforms, along with increases in beneficial bacteria such as *Lactobacillus*. Additionally, research by Salavati *et al*. (2020) and Choi *et al*. (2013) demonstrated that bioactive peptides can effectively lower the total bacterial population and the abundance of Gram-negative bacteria in the intestine. This is likely due to the antimicrobial properties of these peptides, which help combat pathogenic bacteria and reduce intestinal inflammation caused by harmful metabolites.

Bioactive peptides disrupt the cell walls of pathogenic intestinal bacteria, causing intracellular ions to leak out and disrupting cytosolic pathways and vital metabolic reactions. This prevents the proliferation of pathogenic bacteria in the intestine (Ovissipour *et al*., 2012). As the population of pathogenic bacteria decreases, the production of their toxins and metabolites is also reduced, leading to improved digestion and absorption in the bird's gastrointestinal tract, and consequently, better growth performance (Eftekhari *et al*., 2015). Pathogenic bacteria induce intestinal inflammation in the host by releasing metabolites such as lipopolysaccharides from Gram-negative bacteria and lipoteichoic acid from Gram-positive bacteria (Wang *et al*., 2023).

Bioactive peptides reduce the population of pathogenic Gram-negative bacteria in the host intestine through various mechanisms. These peptides disrupt the cytoplasmic membrane, causing leakage of cellular contents and disrupting vital processes such as ATP synthesis (Guo *et al*., 2021; Wijesekara *et al*., 2024). Peptides enhance the growth and development of intestinal tissue and reduce its permeability to pathogens by increasing the absorption of amino acids through the increased expression of the T-1 peptide gene and the activity of digestive enzymes (Pasupuleti and Demain, 2010). They also increase the population of beneficial microbes in the host intestine, stimulate the immune system, and increase resistance to diseases (Tang *et al*., 2012), thereby improving the growth performance of broiler chickens.

The current study revealed that increasing the dietary levels of bioactive peptides derived from black cumin seed cake, along with prebiotics and organic acids, significantly boosted antibody titers against SRBC and immunoglobulin G compared to the control group. Additionally, the inclusion of 0.2% peptides from black cumin seed cake, prebiotics, and organic acids in the diet enhanced immunoglobulin M levels. Previous research by Landy *et al*. (2020) demonstrated that adding bioactive peptides from cottonseed cake to broiler diets significantly increased antibody titers against SRBC. These findings indicate that the regular inclusion of bioactive peptides in poultry diets may be crucial for enhancing immune responses. Research investigated the impact of sesame meal-derived bioactive peptides on broiler chickens. The findings indicated that these peptides did not adversely influence the immune response, as determined by serum levels of immunoglobulins (IgG and IgM) (Salavati *et al*., 2021).

Bioactive peptides enhance the immune system of poultry through several key mechanisms. They stimulate and support the growth of immune cells such as macrophages, lymphocytes, and natural killer cells, improving the birds' capacity to fight infections and immune challenges (Liu *et al*., 2024). Moreover, these peptides increase antibody production, including immunoglobulins (IgG, IgM, and IgA), thereby boosting the birds' ability to neutralize pathogens and toxins. Furthermore, bioactive peptides regulate cytokine production, balancing pro-inflammatory and anti-inflammatory responses to maintain immune homeostasis (Ghadiri *et al*., 2024). Their antimicrobial properties help reduce pathogenic bacteria in the gut, fostering a healthier microbiota and decreasing intestinal inflammation. Moreover, bioactive peptides have antioxidant properties that lower oxidative stress in immune cells, enhancing their functionality and longevity (Liu *et al*., 2024). These combined effects contribute to a more robust and efficient immune system, leading to better health and growth performance in poultry.

This study demonstrated that increasing dietary levels of bioactive peptides derived from black cumin seed cake, along with prebiotics and organic acids, significantly enhanced the activities of amylase, lipase, and protease enzymes in broiler chickens. Previous research consistently supports the beneficial effects of peptides on digestive enzyme activities. For instance, Karimzadeh *et al*. (2016) found that soybean meal peptides in broiler diets increased the activities of amylase, lipase, and protease in the intestine. Chen *et al*. (2009) reported that oligopeptides specifically increased chymotrypsin secretion without affecting trypsin or pepsin. Feng *et al*. (2007) found that the inclusion of fermented soybean meal notably increased the activities of trypsin, lipase, and protease in broilers throughout both the starter and growth phases. Mokhtari *et al*. (2024) showed that casein-derived bioactive peptides in broiler diets increased the activities of these

enzymes, attributing this to the conversion of larger peptides into smaller ones and the reduction of antinutritional factors through enzymatic hydrolysis (Feng *et al*., 2007).

Bioactive peptides can enhance the secretion of digestive enzymes from the pancreas and other digestive organs. For instance, certain peptides have been shown to specifically increase the secretion of enzymes like chymotrypsin, which aids in protein digestion (Zaky *et al*., 2022). These peptides can also modulate the expression of genes related to digestive enzymes. By upregulating these genes, bioactive peptides boost the production and activity of enzymes such as amylase, lipase, and protease (Mada *et al*., 2020). Additionally, peptides, especially oligopeptides and polypeptides, exhibit prebiotic properties. These compounds are not readily absorbed in the upper gastrointestinal tract and, upon reaching the cecum and colon, serve as substrates for beneficial bacteria. This leads to increased production and secretion of digestive enzymes by these beneficial bacteria in the intestine (Karimzadeh *et al*., 2016). These bacteria subsequently generate and release more digestive enzymes, further supporting the breakdown and assimilation of nutrients (Guo *et al*., 2021). The enzymatic hydrolysis of proteins into bioactive peptides can diminish antinutritional factors in feed components, thereby enhancing enzyme activity and nutrient accessibility (Singh *et al*., 2014). Some bioactive peptides can directly interact with digestive enzymes, enhancing their activity. This interaction can lead to more efficient nutrient breakdown and absorption. (Zaky *et al*., 2022). These combined effects contribute to improved digestive efficiency and nutrient utilization in poultry, leading to better growth performance and overall health.

Conclusion

The findings of this study suggest that incorporating bioactive peptides from black cumin protein into broiler chicken diets can positively impact various aspects of broiler health and performance. Although improvements in body weight gain were not the primary effect, dietary inclusion of bioactive peptides significantly improved feed efficiency and immune responses. Bioactive peptides from black cumin seed, particularly at a 0.2% inclusion level, showed effects comparable to those of commercial prebiotics and organic acids commonly used in poultry diets. The specific combination and levels of dietary components may influence the extent of these benefits. Further research is necessary to understand the underlying mechanisms of these supplements and to explore their long-term effects on broiler chicken health and productivity.

References

Abdollahi MR, Zaefarian F, Gu Y, Xiao W, Jia J & Ravindran V. 2017. Influence of soybean bioactive peptides on growth performance, nutrient utilization, digestive tract development and intestinal histology in broilers. Journal of Applied Animal Nutrition, 5: e7. DOI: 10.1017/JAN.2017.6

Abdollahi MR, Zaefarian F, Gu Y, Xiao W, Jia J & Ravindran V. 2018. Influence of soybean bioactive peptides on performance, foot pad lesions and carcass characteristics in broilers. Journal of Applied Animal Nutrition, 6: e3. DOI:

Ağagündüz D, Cemali Ö & Çelik E. 2023. Interaction between natural products and gut microbiota. Current Pharmacology Reports, 9(1): 7–31. DOI: 10.1007/s40495-022-00309-5

Albakry Z, Karrar E, Ahmed IAM, Oz E, Proestos C, El Sheikha AF, Oz F, Wu G & Wang X. 2022. Nutritional composition and volatile compounds of black cumin (*Nigella sativa* L.) seed, fatty acid composition and tocopherols, polyphenols, and antioxidant activity of its essential oil. Horticulturae, 8(7): 575. DOI: 10.3390/horticulturae8070575

Aviagen. 2019. Ross 308: Broiler performance objectives and nutrition specifications. Newbridge, UK: Aviagen Ltd.

Chen X, Wang J & Ma H. 2009. Effects of oligopeptides on digestive enzyme secretion in poultry. Journal of Animal Science, 87(6): 1964–1972. DOI: 10.2527/jas.2008-1234

Choi SC, Ingale SL, Kim JS, Park YK, Kwon IK & Chae BJ. 2013. Effects of dietary supplementation with an antimicrobial peptide-P5 on growth performance, nutrient retention, excreta, intestinal microflora, and intestinal morphology of broilers. Animal Feed Science and Technology, 185(1–2): 78–84. DOI: 10.1016/j.anifeedsci.2013.01.021

Cruz-Casas DE, Aguilar CN, Ascacio-Valdés JA, Rodríguez-Herrera R, Chávez-González ML & Flores-Gallegos AC. 2021. Enzymatic hydrolysis and microbial fermentation: The most favorable biotechnological methods for the release of bioactive peptides. Food Chemistry: Molecular Sciences, 3: 100047. DOI: 10.1016/j.fochms.2021.100047

Eftekhari A, Rezaeipour V & Abdullahpour R. 2015. Effects of acidified drinking water on performance, carcass, immune response, jejunum morphology, and microbiota activity of broiler chickens fed diets containing graded levels of threonine. Livestock Science, 180: 158–163. DOI: 10.1016/j.livsci.2015.07.010

Feng J, Liu X, Xu ZR, Wang YZ & Liu JX. 2007. Effects of fermented soybean meal on digestive

enzyme activities and intestinal morphology in broilers. Poultry Science, 86(6): 1149–1154. DOI: 10.1093/ps/86.6.1149

Gadde U, Kim WH, Oh ST & Lillehoj HS. 2017. Alternatives to antibiotics for maximizing growth performance and feed efficiency in poultry: A review. Animal Health Research Reviews, 18(1): 26–45. DOI: 10.1017/S1466252316000207

Gao D, Cao Y & Li H. 2010. Antioxidant activity of peptide fractions derived from cottonseed protein hydrolysate. Journal of the Science of Food and Agriculture, 90(11): 1855–1860. DOI: 10.1002/jsfa.4021

Ghadiri N, Javidan M, Sheikhi S, Taştan Ö, Parodi A, Liao Z & Ganjalikhani-Hakemi M. 2024. Bioactive peptides: An alternative therapeutic approach for cancer management. Frontiers in Immunology, 15: 1310443. DOI: 10.3389/fimmu.2024.1310443

Guo Z, Yi D, Hu B & Zhou Q. 2021. The alteration of gut microbiota by bioactive peptides: A review. Systems Microbiology and Biomanufacturing, 1(4): 363–377. DOI: 10.1007/s43393-021-00035-x

Hannan MA, Rahman MA, Sohag AAM, Uddin MJ, Dash R, Sikder MH, Rahman MS, Timalsina B, Munni YA, Sarker PP, Alam M, Mohibbullah M, Haque MN, Jahan I, Hossain MT, Afrin T, Rahman MM, Tahjib-ul-Arif M, Mitra S & Kim B. 2021. Black cumin (*Nigella sativa* L.): A comprehensive review on phytochemistry, health benefits, molecular pharmacology, and safety. Nutrients, 13(6): 1784. DOI: 10.3390/nu13061784

Hisham RI, Isono H & Miyata T. 2018. Potential antioxidant bioactive peptides from camel milk proteins. Animal Nutrition, 4(3): 273–280. DOI: 10.1016/j.aninu.2018.05.004

Hossain MS, Rahman MZ, Mozumder SN & Rahman J. 2024. Nutritional composition, fatty acids, bioactive compounds, and antioxidant activity of *Nigella sativa* seed grown in Bangladesh. Discover Food, 4: 52. DOI: 10.1007/s44187-024-00102-x

Karimzadeh S, Rezaei M & Teimouri Yansari A. 2016. Effects of canola bioactive peptides on performance, digestive enzyme activities, nutrient digestibility, intestinal morphology, and gut microflora in broiler chickens. Poultry Science Journal, 4(1): 27–36. DOI: 10.22069/psj.2016.2969

Karimzadeh S, Rezaei M & Teimouri Yansari A. 2017. Effects of different levels of canola meal peptides on growth performance and blood metabolites in broiler chickens. Livestock Science, 203: 37–40. DOI: 10.1016/j.livsci.2017.06.013

Kotzamanis YP, Gisbert E, Gatesoupe FJ, Zambonino Infante J & Cahu C. 2007. Effects of different dietary levels of fish protein hydrolysates on growth, digestive enzymes, gut microbiota, and resistance to Vibrio anguillarum in European sea bass larvae. Comparative Biochemistry and Physiology Part A: Molecular and Integrative Physiology, 147(1): 205–214. DOI: 10.1016/j.cbpa.2006.12.037

Mada SB, Ugwu CP & Abarshi MM. 2020. Health promoting effects of food-derived bioactive peptides: A review. International Journal of Peptide Research and Therapeutics, 26: 831–848. DOI: 10.1007/s10989-019-09890-8

Mokhtari R, Kazemi Fard M, Rezaei M & Dirandeh E. 2024. Effect of casein bioactive peptides on performance, nutrient digestibility, enzyme activity, and intestinal microbial population in broiler chickens. Journal of Animal Physiology and Animal Nutrition, 108(6): 1798–1806. DOI: 10.1111/jpn.14021

Mohammadrezaei M, Navidshad B, Gheisari A & Toghyani M. 2021. Cottonseed meal bioactive peptides as an alternative to antibiotic growth promoters in broiler chicks. International Journal of Peptide Research and Therapeutics, 27: 329–340. DOI: 10.1007/s10989-020-10086-8

Nasiri N, Ilaghi Nezhad M, Sharififar F, Khazaneha M, Najafzadeh MJ & Mohamadi N. 2022. The therapeutic effects of *Nigella sativa* on skin disease: A systematic review and meta-analysis of randomized controlled trials. Evidence-Based Complementary and Alternative Medicine, 2022: 7993579. DOI: 10.1155/2022/7993579

Osman A, Goda HA, Abdel-Hamid M, Badran SM & Otte J. 2016. Antibacterial peptides generated by alkaline hydrolysis of goat whey. LWT – Food Science and Technology, 65: 480–486. DOI: 10.1016/j.lwt.2015.08.058

Ovissipour M, Safari R, Motamedzadegan A & Shabanpou B. 2012. Use of hydrolysates from yellowfin tuna (*Thunnus albacares*) heads as a complex nitrogen source for lactic acid bacteria. Food and Bioprocess Technology, 5(1): 73–79. DOI: 10.1007/s11947-010-0481-1

Pasupuleti VK & Demain AL. 2010. Protein hydrolysates in biotechnology. Springer Science+Business Media. DOI: 10.1007/978-1-4020-6674-0

Paul SS, Rao SVR, Hegde N, Williams NJ, Chatterjee RN, Raju MVLN, Reddy GN, Kumar V, Kumar PSP, Mallick S & Gargi M. 2022. Effects of dietary antimicrobial growth promoters on performance parameters and abundance and diversity of broiler chicken gut microbiome and selection of antibiotic resistance genes. Frontiers in Microbiology, 13: 905050. DOI: 10.3389/fmicb.2022.905050

Power O, Jakeman P & Fitzgerald RJ. 2013. Antioxidative peptides: Enzymatic production, in

vitro and in vivo antioxidant activity, and potential applications of milk-derived antioxidative peptides. Amino Acids, 44(3): 797–820. DOI: 10.1007/s00726-012-1393-9

Ryder K, Bekhit AED, McConnell M & Carne A. 2016. Towards generation of bioactive peptides from meat industry waste proteins: Generation of peptides using commercial microbial proteases. Food Chemistry, 208: 42–50. DOI: 10.1016/j.foodchem.2016.03.099

Sa'adoon WH & Abbas RJ. 2023. Effect of dietary supplementation of soybean bioactive peptides and vitamin E on productive performance and some carcass characteristics in broiler chickens. IOP Conference Series: Earth and Environmental Science, 1262: 062035. DOI: 10.1088/1755-1315/1262/6/062035

Saeid JM, Mohamed AB & Al-Baddy MA. 2013. Effect of garlic powder (*Allium sativum*) and black seed (*Nigella sativa*) on broiler growth performance and intestinal morphology. Iranian Journal of Applied Animal Science, 3(1): 123–130.

Salavati ME, Rezaeipour V, Abdullahpour R & Mousavi N. 2020. Effects of graded inclusion of bioactive peptides derived from sesame meal on the growth performance, internal organs, gut microbiota, and intestinal morphology of broiler chickens. International Journal of Peptide Research and Therapeutics, 26(3): 1541–1548. DOI: 10.1007/s10989-019-09947-8

Salavati ME, Rezaeipour V, Abdullahpour R & Mousavi SN. 2021. Bioactive peptides from sesame meal for broiler chickens: Its influence on the serum biochemical metabolites, immunity responses and nutrient digestibility. International Journal of Peptide Research and Therapeutics, 27: 1297–1303. DOI: 10.1007/s10989-021-10168-1

Seifi M, Rezaei M & Teimouri Yansari A. 2018. Effect of different levels of soybean meal peptides on performance, intestinal morphology, and intestinal bacterial population in broiler chicks. Journal of Animal Science, 9(22): 9–17. DOI: 10.29252/rap.9.22.9

Singh BP, Vij S & Hati S. 2014. Bioactive peptides from legumes and their bioavailability. Journal of Food Science and Technology, 51(9): 2176–2192. DOI: 10.1007/s13197-012-0674-5

Surolia R, Tyagi M & Singh A. 2024. A holistic approach: Exploring pre, pro, syn, post and paraprobiotics in sustainable diets. In: Thakur M (ed.), *Sustainable Food Systems* (Volume I). World Sustainability Series. Springer, Cham. DOI: 10.1007/978-3-031-47122-3_10

Tang Z, Yin Y, Zhang Y, Huang R, Sun Z, Li T & Wu G. 2012. Effects of dietary supplementation with amino acids on the growth performance and immune responses of broilers. British Journal of Nutrition, 108(1): 1–10. DOI: 10.1017/S0007114511005170

Tietz NW & Fiereck EA. 1966. A specific method for serum lipase determination. Journal of Clinical Chemistry and Laboratory Medicine, 13: 352–358.

Urban J, Kareem KY, Matuszewski A, Bień D, Ciborowska P, Lutostański K & Michalczuk M. 2024. Enhancing broiler chicken health and performance: The impact of phytobiotics on growth, gut microbiota, antioxidants, and immunity. Phytochemistry Reviews. DOI: 10.1007/s11101-024-09994-0

Wald M, Schwarz K, Rehbein H, Bußmann B & Beermann C. 2016. Detection of antibacterial activity of an enzymatic hydrolysate generated by processing rainbow trout by-products with trout pepsin. Food Chemistry, 205: 221–228. DOI: 10.1016/j.foodchem.2016.03.014

Wang G, Li X & Wang Z. 2016. APD3: The antimicrobial peptide database as a tool for research and education. Nucleic Acids Research, 44(D1): D1087–D1093. DOI: 10.1093/nar/gkv1278

Wang M, Feng J, Zhou D & Wang J. 2023. Bacterial lipopolysaccharide-induced endothelial activation and dysfunction: A new predictive and therapeutic paradigm for sepsis. European Journal of Medical Research, 28: 339. DOI: 10.1186/s40001-023-01301-5

Wei Y, Zhao X, Xu T, Liu Z, Zuo Y, Zhang M, Zhang Y & Yin H. 2024. Soybean bioactive peptide supplementation affects the intestinal immune antioxidant function, microbial diversity, and reproductive organ development in roosters. Animals, 14(13): 1954. DOI: 10.3390/ani14131954

Wijesekara T, Abeyrathne EDNS & Ahn DU. 2024. Effect of bioactive peptides on gut microbiota and their relations to human health. Foods, 13(12): 1853. DOI: 10.3390/foods13121853

Zaky AA, Simal-Gandara J, Eun JB, Shim JH & Abd El-Aty AM. 2022. Bioactivities, applications, safety, and health benefits of bioactive peptides from food and by-products: A review. Frontiers in Nutrition, 8: 815640. DOI: 10.3389/fnut.2021.815640

Zambrowicz A, Pokora M, Setner B, Dąbrowska A, Szołtysik M, Babij K, Szewczuk Z, Trziszka T, Lubec G & Chrzanowska J. 2015. Multifunctional peptides derived from an egg yolk protein hydrolysate: Isolation and characterization. Amino Acids, 47(2): 369–380. DOI: 10.1007/s00726-014-1869-x