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Research article

The effect of subtilisin-like serine protease *Bacillus pumilus* and histidine acid 3-phytase *Pantoea brenneri* on growth performance, digestibility, immune organs, and expression of intestinal barrier genes in broiler chickens

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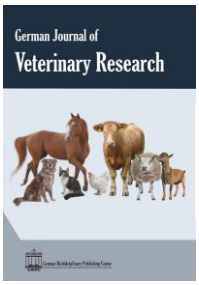

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Abstract

The study determined the effect of subtilisin-like protease *Bacillus pumilus* and phytase *Pantoea brenneri* on poultry growth performance, digestibility, digestion of feed nutrients, changes in immune organs weight, as well as on differential gene expression in the ileum tissues of broilers. A total of 240 Ross 308 broilers were divided into four groups: one control and three experimental groups. Each group contained 60 birds, organized into four replicates of 15 birds each. Groups received four feed rations from 0 to 35 days. The diet of the control group was a basal diet (BD) without additives. In the experimental groups, the BD was supplemented with one of the additives: protease (10 U/kg), phytase (1000 FTU/kg), or their combination. The results showed that decreasing the addition of phytase and protease to feed increased body weight and average daily gain of broilers (p<0.05) while reducing the feed conversion ratio (p<0.0001). Enzyme supplementation increased calcium, phosphorus, and nitrogen availability and improved protein, fiber, and fat digestibility (p<0.002). No significant differences were found in the weight of the liver, spleen, thymus, and bursa of Fabricius in the experimental and control groups (p>0.05). The gene expression of pro-inflammatory cytokines on day 14 of feeding was lower in the groups receiving protease and phytase compared to the control group (p<0.0001). In the case of MUC2 and Occludin genes, increased expression was observed in the group receiving protease (p<0.05). In contrast, decreased expression was observed for the groups receiving phytase and combined feed additive. Junctional adhesion molecule-2 (JAM2) gene expression decreased for all experimental groups receiving enzyme feed additives (p<0.05). Thus, it was established that adding protease and phytase to the BD for broilers enhances the digestibility of feed components, reduces the amount of feed consumed, doesn't affect the weight of liver and lymphoid organs, and reduces inflammation in the ileum of chickens. At the same time, protease supplementation increases the mucin and tight contact protein gene expression, which may indicate an improvement in the ileum of broiler chickens.

Keywords: Protease, Phytase, Poultry, Feed additive, Intestinal barrier genes, Immune response

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Introduction

A topical problem of agricultural production is to obtain economic benefits from a more complete digestion of grain feed in poultry production by increasing the digestibility of nutrients of feed components. Enzyme-based feed additives can solve this problem. Feed additives are used to increase nutrient absorption and reduce toxicity and bacterial contamination of feeds (Borda et al., 2019). Several enzymes are used in the poultry industry to enhance feed digestibility (Singh et

al., 2021; Azzaz et al., 2021; Koryagina et al., 2021; Bulmakova et al., 2020). The possibility of using microbial proteases in poultry production is due to their ability to tolerate harsh conditions and exhibit stability and broad substrate specificity in the digestive system of broilers (Lourenco et al., 2020). The primary purpose of adding proteases to poultry feed is to improve the availability of amino acids; exogenous proteases also contribute to increased digestibility of starch and fat (Cupi et al., 2022). It has been shown that exogenous proteases not only complement animal digestive enzymes such as trypsin and pepsin but they also break down lectins and trypsin inhibitors (Cowieson and Adeola, 2005; Cupi et al., 2022).

Research has shown that adding proteases to poultry feed improves poultry growth performance (Borda et al., 2019) and positively affects intestinal microbiota composition (Giannenas et al., 2017). One enzyme widely used as a feed additive in poultry farming is phytase (Abd El-Hack et al., 2018). The chicken feed compound is based on grain that contains an increased amount of hard-to-digest phytate, which holds up to 80% of the total phosphorus content of the grain (Vieira et al., 2016). Most of the phytate ingested into the body passes through the gastrointestinal tract of chickens in an unabsorbed form, causing environmental pollution and eutrophication of water reservoirs (Abd El-Hack et al., 2018). The use of phytases that break down phytate can increase the availability of myo-inositol and phosphate in feeds (Thorsen et al., 2021), reducing phosphorus accumulation in the litter, as well as increasing the bioavailability of many minerals and proteins that would otherwise be bound to phytate (Dersiant et al., 2015).

Today, there is an active search for new effective enzymes to expand the arsenal of commercially available additives already on the market. The subtilisin-like serine protease of *B. pumilus* 7P has broad substrate specificity and is most active at pH 10.0–11.5 and at the optimum temperature of 55°C (Mikhailova et al., 2009). It was shown that chicken bile at a concentration of 0.01–0.05% and trypsin inhibitors did not affect the protease activity, making it a potential candidate for use as a feed additive (Koryagina et al., 2018). Intracellular histidine acid 3-phytase AgpP of *P. brenneri* 3.5.1 has broad substrate specificity, is stable at pH 3.0–6.0, and at temperatures from 10 to 45°C, which also allows it to be used in feeding broilers (Suleimanova et al., 2015). Previously, the positive effects of these feed additives on the productivity, blood parameters, and histological parameters of Hubbard broiler chickens were described (Koryagina et al., 2021; Bulmakova et al., 2020). One factor in assessing a particular feed's usefulness is its immunogenicity. Dynamic analysis of immune organs' weight and expression of marker genes of immune response allows for evaluating the effect of feed additives on the state of animal immunity.

The study aimed to evaluate the impact of feed additives based on *B. pumilus* 7P protease and *P. brenneri* 3.5.1 phytase on the growth performance, nutrient digestibility, immune organ weight of Ross308 broiler chickens, and differential gene expression in ileum tissues such as mucin synthesis gene (MUC2), cytokine genes (IL-8, IL-17F, TNFSF15), and intestinal barrier genes (JAM2, Occludin, ZO-1).

Material and methods

Preparation of feed additives

The study used the subtilisin-like protease of *B. pumilus* 7P and histidine acid phytase of *P. brenneri* 3.5.1 as feed additives. Extracellular serine protease was isolated from the nutrient medium of *B. pumilus* 7P (Mikhailova et al., 2009).

At 24 h of cultivation, the cells of *B. pumilus* were removed by centrifugation. Chromatography on carboxymethyl cellulose (Sigma-Aldrich, Burlington, Massachusetts, USA) was used to purify the enzyme. To obtain a large amount of the *P. brenneri* 3.5.1 phytase, the *agpP* gene was cloned and expressed in *Pichia pastoris* (named AgpP-P). AgpP-P was extracted from the cell-free culture medium of transformed *P. pastoris* yeast induced with methanol for 36 h. The recombinant phytase was purified on Ni-NTA agarose (Qiagen, Hilden, Germany) (Suleimanova et al., 2020). The enzyme concentration was expressed in units of activity per kg of feed. One unit of activity was defined as the amount of enzyme necessary to change the absorbance by 1 optical density unit per minute.

Conditions for keeping broilers

The birds were kept following the guidelines for the Care and Use of Experimental Animals of the Kazan Federal University and the Poultry

Industrial Farm (KFK Alimchueva Z.I., Russia, Mari El Republic, Medvedevskiy District, Sredneye Azyakovo village); as well as the Directive of the European Parliament and of the Council on the protection of animals used for scientific purposes of 22 September 2010 (Directive 2010/63/UE on the protection of animals used for scientific purposes). All animal management and experimental procedures for this study were approved by the Local Ethics Committee of KFU (Permit number: 22).

A total of 240 one-day-old broiler chickens (Ross308) with an initial average weight of 54.5±0.6 g were purchased. Birds were labeled using plastic rings of different colors and randomly divided into four groups, each with 60 birds. One control and three experimental groups, with four replicates in each group (15 birds per replicate). We involved both male and female birds in equal proportions for the study. The birds were allocated to 16 battery cages (15 birds per cage) with a controlled temperature regime. The initial temperature was 32°C during the first week and then decreased until reaching 18°C at the end of the experiment. A 23-hour light period per day was provided throughout the experiment. The chicks had free access to water and feed throughout the experimental period. Observations on the general condition of the birds, water, feed, temperature, light, litter condition, and mortality were recorded twice daily. Room temperature and relative humidity were recorded daily and adjusted accordingly to avoid the influence of stressful conditions on broiler chickens.

Dietary procedures

Ross308 broiler chickens were randomly sorted into one of four dietary groups: I – control group fed with a basal diet (BD), II – experimental group was fed the BD supplemented with protease (10 U/kg), III – experimental group received the BD with phytase (1000 FTU/kg), and IV– experimental group received both dietary supplements (10 U/kg protease+1000 FTU/kg phytase). The experimental groups received BD with enzymes from the 6th day of growth. The broilers were fed a three-phase diet with dry compound feed, with nutritional values corresponding to the recommended feeding standards (GOST 18221-99 "Compound feed for poultry. Specifications"). Starter diets were offered to the broilers from days 0 to 10, grower diets from days 11 to 21, and finisher diets from days 22 to 35. The ingredients and the nutrient contents of the broiler feed are shown in Table 1.

Table 1: Ingredient and nutrient contents of broiler chickens' diet.

Ingredient	**Days**		
	0–10	**11–21**	**22–35**
ME (kcal/100g)	305	307	311
Crude fat (%)	5.15	5.99	6.48
Crude protein (%)	23.0	21.7	18.0
Crude fiber (%)	3.55	3.73	4.77
Lysine (%)	1.43	1.32	1.08
Methionine and cysteine (%)	1.08	1.01	0.86
Threonine (%)	0.98	0.90	0.77
P (%)	0.83	0.82	0.74
Digestible P (%)	0.48	0.48	0.4
Ca (%)	1.0	0.9	0.9
K (%)	0.76	0.71	0.60
Na (%)	0.17	0.20	0.15
Cl (%)	-	-	0.2
Vitamin-mineral premix[1] (%)	0.5	0.5	0.5

[1]Containing by kg of diets (for 0-10 and 11-35 days of age): Vitamin A 14.40 and 12.00 IU*10^3, Vitamin D_3 4.8 and 4.00 IU*10^3, Vitamin E 72.00 and 60.00 mg, Vitamin K_3 2.40 and 2.00 mg, Vitamin B_1 2.40 and 2.00 mg, Vitamin B_2 9.60 and 8.00 mg, Vitamin B_3 36.00 and 30.00 mg, Vitamin B_4 600.00 and 500.00 mg, Vitamin B_5 12.00 and 10.00 mg, Vitamin B_6 3.60 and 3.00 mg, Vitamin B_{12} 0.03 and 0.025 mg, Vitamin H 0.12 and 0.10 mg, Fe 30.00 and 25.00 mg, Cu 12.00 and 10.00 mg, Zn 96.00 and 80.00 mg, Mg 96.00 and 80.00 mg, Co 1.20 and 1.00 mg, I 0.84 and 0.70 mg

Growth performance, digestibility, and absorption of nutrient indicators

The body weight of the chickens was measured every 7 days throughout the experiment. Average daily gain (ADG) and feed conversion ratio (FCR) were calculated at 35 days of age. Feed intake (FI) was evaluated weekly and re-estimated for a single bird. A balance experiment was conducted to study the effect of enzyme additives on the

digestibility and assimilation of nutrients (protein, dry matter, fiber, fat, calcium, phosphorus, and nitrogen). To conduct the experiments, the birds were kept in cages with a mesh bottom, under which frames of polyethylene film were installed to collect feces. Comparative quantitative and chemical analysis of feed and bird feces was performed using the methods described in GOST 31640-2012 (dry matter), GOST 32933-2014 (crude ash), GOST 31675-2012 (crude fiber), GOST R 50852-96 (calcium and phosphorus) and using the Kjeldahl (nitrogen), Soxhlet (fat) extraction methods. The digestibility coefficient was calculated as the ratio of digested and consumed nutrients, expressed as a percentage.

Sample collection

For organ weight measurements, six birds of control and experimental groups on days 14 and 35 after hatching were weighed and sacrificed by cervical dislocation after a two-hour fast. The weight of the liver, spleen, thymus, and bursa of Fabricius was determined relative to the total live body weight (Relative organ weight = organ weight/body weight×100). Three birds were selected from each group after 14 days to collect ileum samples to determine the expression of intestinal barrier genes. Intestinal slice samples (triplicate) were collected and stored in RNA preservation and stabilization buffer (RNAlater®, Applied Biosystems, Waltham, Massachusetts).

RNA isolation and cDNA synthesis

The ileum tissue samples were cryogenically minced using liquid nitrogen. Total RNA from broiler chicken ileum tissue was isolated using the phenol method using TRIzol Reagent (ThermoFisher Scientific, Waltham, Massachusetts), following the manufacturer's instructions. Total RNA quantity and purity were measured using a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific, Waltham, Massachusetts). Before cDNA synthesis, RNA samples were treated with DNase (ThermoFisher Scientific, Waltham, Massachusetts). In this procedure, 1 µL of 10× buffer (with $MgCl_2$) and 1 µL of DNAase were added to 1 µg of sample and brought to 10 µL with DEPC-water (ThermoFisher Scientific, Waltham, Massachusetts). This mixture was incubated at 37°C for 30 min. Then 1 µL of 50 mM EDTA was added and incubated for 10 min at 65°C. Extracted RNA from each sample was subjected to reverse transcription using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher Scientific, Waltham, Massachusetts) according to the manufacturer's instructions. Briefly, 10 µL of purified extracted RNA from each sample was mixed with reverse transcription reaction components containing 1 µL of MultiScribe reverse transcriptase, 2 µL of 10× RT buffer, 0.8 µL of 25× dNTP Mix, 2 µL of 10× Random Primers mix, and 4.2 µL of DEPC-water. The reaction was performed at 25°C for 10 min, 37°C for 120 min, and 85°C for 5 min to convert RNA to cDNA. cDNA was stored at -25°C until needed. The primers used for real-time PCR, listed in Table 2, were sourced from previously published studies on chickens (Gadde et al., 2017).

Table 2: Sequences of primers used for quantitative real-time polymerase chain reaction.

Gene	Protein name	Primer sequence (5'–3')	Size (bp)
IL–8	Interleukin 8	F-GGCTTGCTAGGGGAAATGA R-AGCTGACTCTGACTAGGAAACTGT	200
IL–17F	Interleukin 17F	F-TGAAGACTGCCTGAACCA R-AGAGACCGATTCCTGATGT	117
TNFSF15	TNF Superfamily Member 15	F-CCTGAGTATTCCAGCAACGCA R-ATCCACCAGCTTGATGTCACTAAC	292
Occludin	Occludin	F-GAGCCCAGACTACCAAAGCAA R-GCTTGATGTGGAAGAGCTTGTTG	68
ZO–1	Zonula occludin 1	F-CCGCAGTCGTTCACGATCT R-GGAGAATGTCTGGAATGGTCTGA	63
JAM2	Junctional adhesion molecule 2	F-AGCCTCAAATGGGATTGGATT R-CATCAACTTGCATTCGCTTCA	59
MUC2	Mucin 2	F-GCCTGCCCAGGAAATCAAG R-CGACAAGTTTGCTGGCACAT	59
ACTB	Beta (β)-actin	F-ATCCGGACCCTCCATTGTC R-AGCCATGCCAATCTCGTCTT	120

Real-time quantitative PCR

A dynamic analysis of the expression of immune response genes was conducted to assess feed additives' effect on broilers' immune status. Real-time PCR was performed to determine the differential expression of mRNA transcripts of cytokines (IL-8, IL-17F, TNFSF-15), intestinal barrier proteins (JAM2, Occludin, ZO-1), and mucin MUC2 in ileal DNA samples. The beta-actin (ACTB) gene was selected as a housekeeping gene. Quantitative PCR was performed using a ready-to-use PCR mix qPCRmix-HS SYBR (Eurogen, Moscow, Russian Federation). The PCR reaction was 20 µL volume containing 0.4 µM of each primer, 10 µL of qPCRmix-HS SYBR (1×) (Eurogen, Moscow, Russian Federation) and 1 µL of cDNA. All reactions were carried out in three replicates using the protocol: 95 °C for 3 min, followed by 40 cycles at 95 °C for 10 sec and 60 °C for 30 sec. PCR results were analyzed using the $2^{-\Delta\Delta Ct}$ method based on cycle threshold (Ct) values using ACTB gene cDNA as an internal control. The ΔCt value was calculated by subtracting the mean Ct value of the ACTB gene cDNA from the mean Ct value of the target gene. Then, the ΔΔCt value was calculated by subtracting the ΔCt values of the control group (without feed additives) from the experimental groups.

Statistical analyses

Statistical processing was performed in GraphPad Prism version 8.4.3 (Dotmatics, Bishop's Stortford, United Kingdom) and R version 4.4.1 using the RStudio graphical shell (ggplot2, northeast, car packages). Data were tested for normality and homogeneity of variance using the Shapiro-Wilk and Fisher tests. All variables were normally distributed and compared using one-way ANOVA and Tukey's test for multiple pairwise comparisons of groups. Results are presented as mean ± standard error of the mean (SEM), with $p<0.05$ considered significant.

Results and discussion

Digestibility of nutrients and productivity

The effect of enzymatic feed additives on body weight changes of Ross 308 broiler chickens was studied. In the first week of life (1-7 days), when chickens were fed BD with additives, no significant differences in the body weight of control and experimental groups were observed. At 14 days of age, the body weight of broilers of group II was higher by 5.8%, III by 5.4%, and IV by 6.72% relative to the control group ($p=0.005$). In the following weeks, the highest bird weight was recorded in experimental groups II and IV ($p<0.05$), possibly resulting from using protease enzymes in both groups. By the end of the experiment, on the 35th day, the weight of chickens of experimental group II was higher by 19.2%, III by 14.9%, and IV by 19.5%, relative to the control group ($p<0.05$). The feed conversion ratio (FCR) of the experimental groups was 2.087±0.004 (for II), 2.098±0.003 (for III), and 2.065±0.003 (for IV), which was lower than the FCR value of the control group (2.344±0.004) by 10.9%, 10.5% and 11.9% ($p=0.0001$).

During the experiment, the survival rate of birds of all groups amounted to 100%, and no diseases were detected during veterinarian examination. Average daily gain (ADG) in chickens of experimental groups II, III, and IV amounted to 43.02±1.69 g, 41.44±1.75 g, and 43.13±1.92 g, respectively, and was significantly higher than the control (35.85±1.78 g) ($p=0.003$) (Table 3).

Table 3: Growth performance based on changes in Ross 308 broilers weight (g).

Indicator	Control group (BD[1])	Experimental group II (BD[1]+protease)	Experimental group III (BD[1]+phytase)	Experimental group IV (BD[1]+protease+phytase)	*p*-value
0 w[2]	54.5[a]±0.7	54.5[a]±0.7	54.5[a]±0.6	54.5[a]±0.6	0.963
1 w[2]	188.9[a]±3.7	195.1[a]±3.0	190.9[a]±2.8	191.8[a]±2.4	0.542
2 w[2]	434.3[a]±6.9	459.5[b]±6.6	457.8[b]±4.7	463.5[b]±6.4	0.005
3 w[2]	731.2[a]±9.6	762.8[b]±11.8	749.8[ab]±13.3	770.2[b] ±14.3	0.013
4 w[2]	1058.0[a]±31.2	1185.0[b]±25.7	1152.0[b]±31.7	1199.0[b]±32.8	0.006
5 w[2]	1309.0[a]±62.9	1560.0[b]±58.9	1505.0[b]±61.2	1564.0[b]±67.0	0.016
ADG (g)	35.85[a]±1.78	43.02[b]±1.69	41.44[b]±1.75	43.13[b]±1.92	0.003
FI (g)	2941.05[a]±141.3	3140.44[a]±118.57	3042.92[a]±123.7	3117.57[a]±133.6	0.301
FCR	2.344[a]±0.004	2.087[b]±0.004	2.098[c]±0.003	2.065[d]±0.003	0.0001

BD[1] = basal diet; w[2] =week; Date presented as the mean±SEM. [a,b,c,d] denote significant differences ($p<0.05$).

Several studies have shown an increase in poultry weight gain and improvement in feed conversion ratio after the addition of phytase and protease enzymes (Mohammadigheisar and Kim, 2018; Pirgozliev et al., 2010; De Sousa et al., 2015). On the contrary, some studies demonstrated that including protease or phytase in feed did not improve broiler growth performance, feed intake, and nutrient digestibility (Walk et al., 2019; Khan et al., 2019).

A balance experiment was conducted to study the effect of protease and phytase on digestibility and assimilation of nutrients of mixed fodder by broiler chickens Ross 308 (Table 4). The highest calcium (Ca) digestibility coefficient was found in chickens of experimental group IV, which received the combined feed additive (60.78±0.117%), and the lowest in the control group chickens (56.06±0.283%). At the same time, the value of the calcium digestibility coefficient in group III was close to the value of group IV (60.18±0.707%), which may indicate the effect of phytase on calcium assimilation. Phytase application also had a positive impact on phosphorus (P) digestion. The phosphorus digestibility coefficient in experimental groups III and IV chickens was 40.16±0.248% and 41.36±0.343%, respectively, while in control was 29.94±0.633%. The increase in Ca and P availability is because phytase releases these minerals from the Ca-phytate complex, making them available for broilers to digest (Chung et al., 2013). At the same time, phosphorus excretion was significantly reduced in groups III and IV, which is essential for lowering P pollution from poultry manure. Other researchers also confirmed the reduction in phosphorus excretion when phytase was added to the diet (Kanagaraju et al., 2013).

Protease application influenced nitrogen assimilation. The highest digestibility coefficient was found in chickens of experimental groups II (56.7±0.15%) and IV (57.72±0.217%). This result is due to increased protein digestion when exogenous protease was used, which was reflected in the nitrogen balance. Other researchers have also shown that adding protease to feed decreased protein loss and nitrogen excretion levels (Murugesan et al., 2015). Thus, enzyme complexes increased the assimilation of nitrogen, phosphorus, and calcium from feed by broiler chickens (Table 4).

Table 4: Effect of supplements on absorption of calcium, phosphorus, and nitrogen.

Parameters	Control group (BD[1])	Experimental group II (BD[1]+protease)	Experimental group III (BD[1]+phytase)	Experimental group IV (BD[1]+protease+phytase)	*p*-value
			Calcium		
Ingested, g	1.208[a]±0.001	1.212[a]±0.001	1.212[a]±0.001	1.21[a]±0.001	0.99
Excreted, g	0.53[a]±0.0001	0.496[b]±0.0001	0.482[c]±0.0001	0.474[d]±0.0001	<0.0001
Digested, g	0.678[a]±0.0001	0.716[b]±0.0001	0.73[c]±0.0002	0.736[c]±0.0001	<0.0001
Digestibility, %	56.06[a]± 0.283	59.08[b]±0.827	60.18[c]±0.707	60.78[c]±0.117	<0.0001
			Phosphorus		
Ingested, g	0.75[a]±0.0001	0.75[a]±0.0001	0.746[a]±0.0001	0.746[a]±0.0001	0.344
Excreted, g	0.524[a]±0.0001	0.526[a]±0.0001	0.446[b]±0.0001	0.426[c]±0.0001	<0.0001
Digested, g	0.226[a]±0.00004	0.23[b]±0.000025	0.3[c]±0.0001	0.32[d]±0.00005	<0.0001
Digestibility, %	29.94[a]±0.633	30.28[b]±0.437	40.16[c]±0.248	41.36[d]±0.343	<0.0001
			Nitrogen		
Ingested, g	3.148[a]±0.0001	3.148[a]±0.0001	3.15[a]±0.0001	3.152[a]±0.0001	0.913
Excreted, g	1.468[a]±0.0001	1.348[b]±0.0001	1.426[c]±0.0001	1.33[d]±0.0001	<0.0001
Digested, g	1.676[a]±0.0001	1.792[b]±0.0001	1.742[c]±0.0001	1.816[d]±0.0001	<0.0001
Digestibility, %	53.36[a]±0.063	56.7[b]±0.15	54.58[c]±0.287	57.72[d]±0.217	<0.0001

BD[1] =basal diet; Date presented as the mean±SEM. [a,b,c,d] denote significant differences (p<0.05).

In the current study, the coefficients of organic matter digestibility for broiler chickens of the control group and groups were calculated by adding exogenous enzymes (Table 5). The increase of organic matter digestibility in the diet of broilers of the experimental groups was mainly due to the improvement of fat and protein digestibility. The highest protein digestibility was observed in experimental group IV, which received the combined feed additive (82.6±0.55%), which was 9% higher compared to the control group. The improvement in protein digestibility was mainly due to protease utilization. Freitas showed that serine protease supplementation positively affected the feed-to-body weight gain ratio and improved the digestibility of crude protein and fat (Freitas et al., 2011). Dry matter digestibility in chickens of the control group was 72.6±1.14%.

In contrast, the experimental group IV was 75.4±1.14%, which may result from using phytase as part of the combined preparation. Adding phytase to broiler feed has been shown to significantly increase the digestibility of dry matter and protein (Moita et al., 2021) and does not affect fat digestibility (Ptak et al., 2013). In experimental group IV, the digestibility coefficient of fiber (13.6±0.3%) and fat (60.8±1.7%) was also higher than in other groups (Table 5). Thus, adding protease and phytase enzymes to the diet improved the digestibility of feed nutrients, as evidenced by the increase in digestibility coefficients in the experimental groups compared to the control.

Effect of feed additives on the growth of immune system organs of broiler chickens

The weight of Fabricius's liver, spleen, thymus, and bursa was measured to investigate the effect of enzyme feed additives on poultry health. In our study, no significant differences in liver (p=0.399) and spleen (p=0.259) weight were observed in the experimental groups on the 14th day of bird growth (Figure 1).

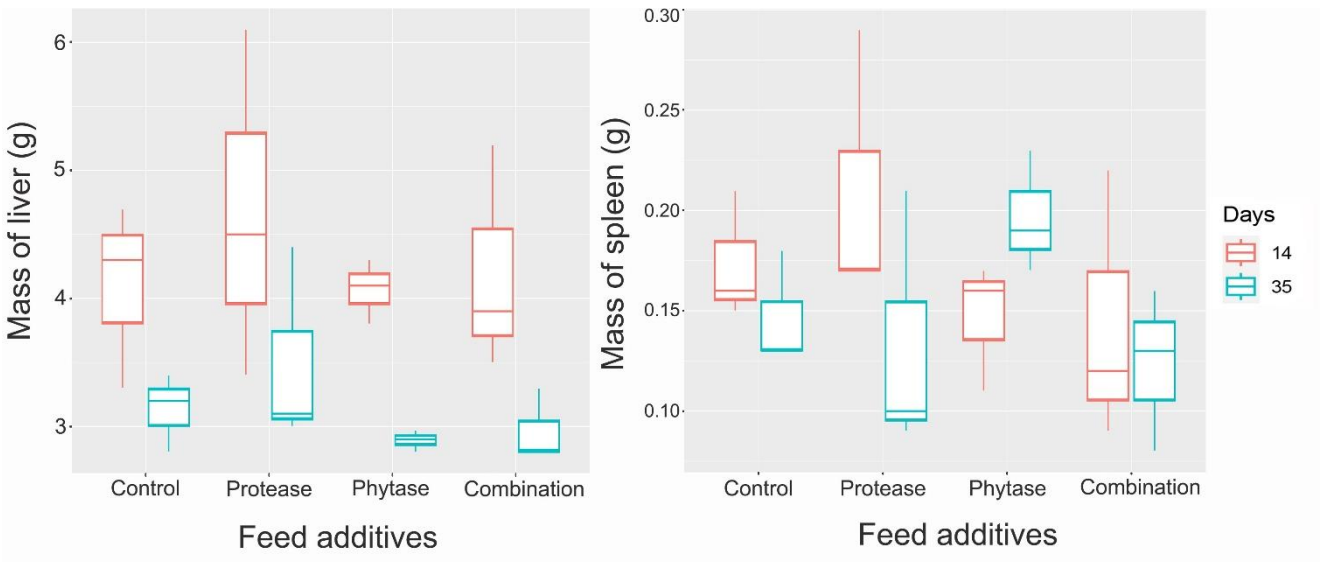

Figure 1: Liver and spleen weight for four groups of chickens at 14 (red) and 35 (green) days.

Table 5: Effect of the different diets on nutrient digestibility.

Digestibility coefficient (%)	Control group (BD[1])	Experimental group II (BD[1]+protease)	Experimental group III (BD[1]+phytase)	Experimental group IV (BD[1]+protease+phytase)	*p*-value
Protein	75.2[a]±0.83	78.8[b]±0.84	76[c]±0.71	82.6[d]±0.55	<0.0001
Dry matter	72.6[a]±1.14	72.8[a]±0.83	73.6[b]±0.89	75.4[c]±1.14	0.002
Fiber	11.4[a]±0.8	12.8[b]±0.7	13[b]±0.5	13.6[c]±0.3	0.002
Fat	54.4[a]±1.3	59.2[b]±1.7	59.4[b]±1.3	60.8[c]±1.7	<0.0001

BD[1] – basal diet; [a,b,c,d] - mean values in the row marked with superscripts differed significantly from each other (p<0.05); the results were presented as the mean ± SEM.

On the 35th day, the relative spleen weight of the control group was 0.15±0.028, in experimental groups II was 0.13±0.067, III was 0.2±0.031, IV was 0.12±0.04 (p=0.369). The values of relative liver weight on the 35th day were also not significantly different (p=0.399). They were 3.13±0.3 for the control group, 3.5±0.79 for the group supplemented with protease, 2.89±0.085 for the group supplemented with phytase, and 2.97±0.29 for the group supplemented with two additives. Previous reports on enzyme feed supplements' effects on broilers' organ weights have provided conflicting data. Park and Kim showed that protease and essential oils-supplemented diets had no differences in the liver, bursa of Fabricius, and spleen relative weights compared to the control group (Park and Kim, 2018). Omojola and Adesehinwa also showed that enzyme inclusion had no effect (p>0.05) on the relative weights of kidneys, hearts, and livers of all birds (Omojola and Adesehinwa, 2007).

Conversely, experiments with various proteases have shown that the dietary supplement promoted significant liver enlargement (Rehman et al., 2018). Wang et al. showed that phytase supplementation increased liver (p<0.05) and spleen (p<0.05) weights (Wang et al., 2013). However, Akyurek et al. reported that phytase supplementation to a low-phosphorus diet did not affect heart, liver, and spleen weights (Akyurek et al., 2011).

The primary immune organs in birds are the bursa of Fabricius, where B-lymphocyte hematopoiesis occurs, and the thymus, where thymopoiesis occurs (Kaiser and Balic, 2015). The normal development of these organs is a crucial indicator of a bird's health. In our study, we observed no significant differences in the weights of the thymus (p=0.097) and the bursa of Fabricius (p=0.233) between the groups that consumed additives and the control group on the 35th day of growth (Figure 2). Thus, protease and phytase additives did not affect the weight of organs either when consumed separately or when consumed together by broilers.

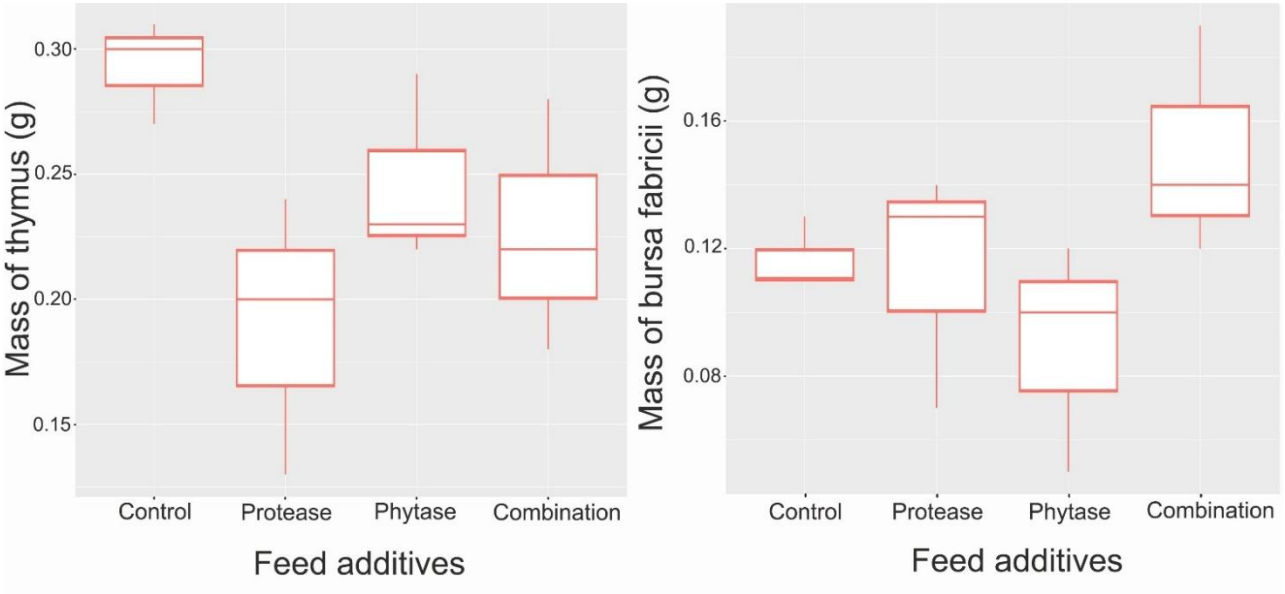

Figure 2: Thymus and bursa of Fabricius weight for four groups of chickens on day 35.

Relative mRNA Expression of pro-inflammatory cytokine genes

To determine the effect of enzyme additives on the intestinal health of chickens, it is essential to assess the normal formation of the intestine in the first days of the chicken's life. One of the criteria for intestinal health is the absence of inflammatory reactions and the normal formation of intercellular contacts in the epithelial tissues. The expression level of mRNA of pro-inflammatory cytokine genes (IL-8, IL-17F, TNFSF-15) was assessed in the ileum tissue of broilers whose diets were supplemented with bacterial enzymes. Using the β-actin gene as a reference, it was found that the expression of pro-inflammatory cytokine genes (IL-8, IL-17F, TNFSF-15) on the 14th day of feeding was 8 times lower for the groups consuming feed additives based on protease and phytase compared to the control group (p<0.0001) (Figure 3).

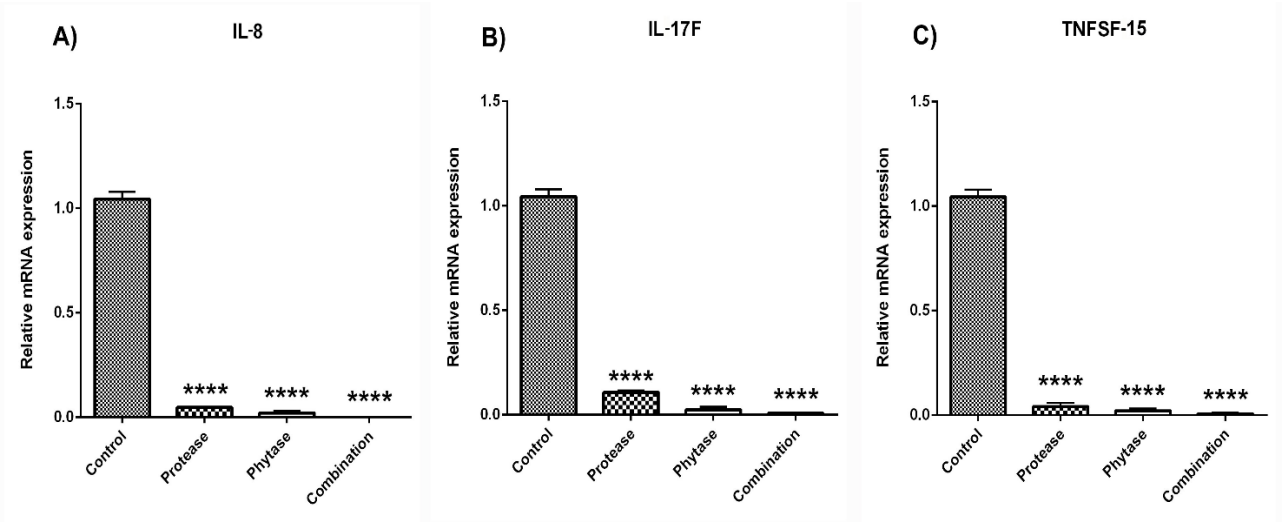

Figure 3: Effect of enzyme feed additives on the expression of pro-inflammatory cytokine genes in the ileum of broiler chickens. Panels A–C shows the expression of IL-8, IL-17F, and TNFSF15 genes in the ileum by real-time qPCR. Data are presented as mean with SEM (N = 3). Superscript asterisks on the bars indicate significant differences in comparing treatment to the control group (*, p<0.05; **, p<0.01; ***, p<0.001; ****, p<0.0001).

Interleukins IL-8 and IL-17F are the most critical pro-inflammatory cytokines (Dobreva et al., 2006; Cai et al., 2011). A decrease in the expression of the IL-8 and IL-17F genes on the 14th day of feeding with the subtilisin-like protease *B. pumilis* 7P and histidine phytase *P. brenneri* 3.5.1 compared to the control group indicates a decrease in the inflammatory reaction in the ileum of chickens from the experimental groups. TNFSF-15 (VEGI) also plays a vital role in stimulating the body's immune response, as VEGI stimulates the growth of dendritic cells (Sethi et al., 2009). Thus, a decrease in the expression of the TNFSF-15 gene also indicates that the additives reduce the immune response in the intestines of broilers.

The effects obtained are more pronounced when using food additives together. This may be because the mechanisms that reduce the immune response are different for each additive and complement each other in the combination preparation. Indeed, the anti-inflammatory effect of proteases is associated with the breakdown of allergens contained in food (Zhang et al., 2018; Cheng et al., 2019), which is why the use of proteases is often accompanied by a decrease in the expression of pro-inflammatory cytokines (Cheng et al., 2019). Using phytase as a feed additive also led to a reduction in the expression of pro-inflammatory cytokines. Phytates, broken down by phytases, are a significant group of antinutritional compounds (Thorsen et al., 2021; Selle et al., 2007). Phytates can bind proteins (Selle et al., 2007), including digestive enzymes, which reduces the ability of the chicken's body to break down allergens. Therefore, combining phytase and protease may be more effective in reducing the immune response than using them separately.

Relative mRNA expression of genes related to tight junction proteins

The intestinal mucosa plays a vital role in animals as a barrier to prevent the penetration of pathogens and toxins. The complex structure of the intestinal barrier consists of four main components: chemical, physical, immunological, and microbiological barriers (Anderson et al., 2012).

The MUC2 protein is the main component of the chemical barrier, which plays a vital role in intestinal mucosal immunity since the breakdown of pathogens occurs only after their binding to the polysaccharide chains of mucins (Johansson et al., 2008). In the case of MUC2, an increased (compared to the control group) expression was observed in the II experimental group (by 2.04 times, p<0.05) and a decreased one for the group of chickens receiving the combined preparation (p<0.05) (Figure 4, A). For the group of chickens receiving phytase as a feed additive, no significant differences in the expression of the MUC2 gene were found (p>0.05). The increased expression of the MUC2 gene in the protease-supplemented group may be due to higher amino acid availability. It is known that under inflammatory conditions, the

increased amino acid content in the diet can promote mucin synthesis, balance the gut microbiota, and thus promote colon protection and mucosal healing (Faure et al., 2006). Therefore, the increased expression of MUC2 in protease-supplemented chickens compared to other groups may indicate improved gut health in this group.

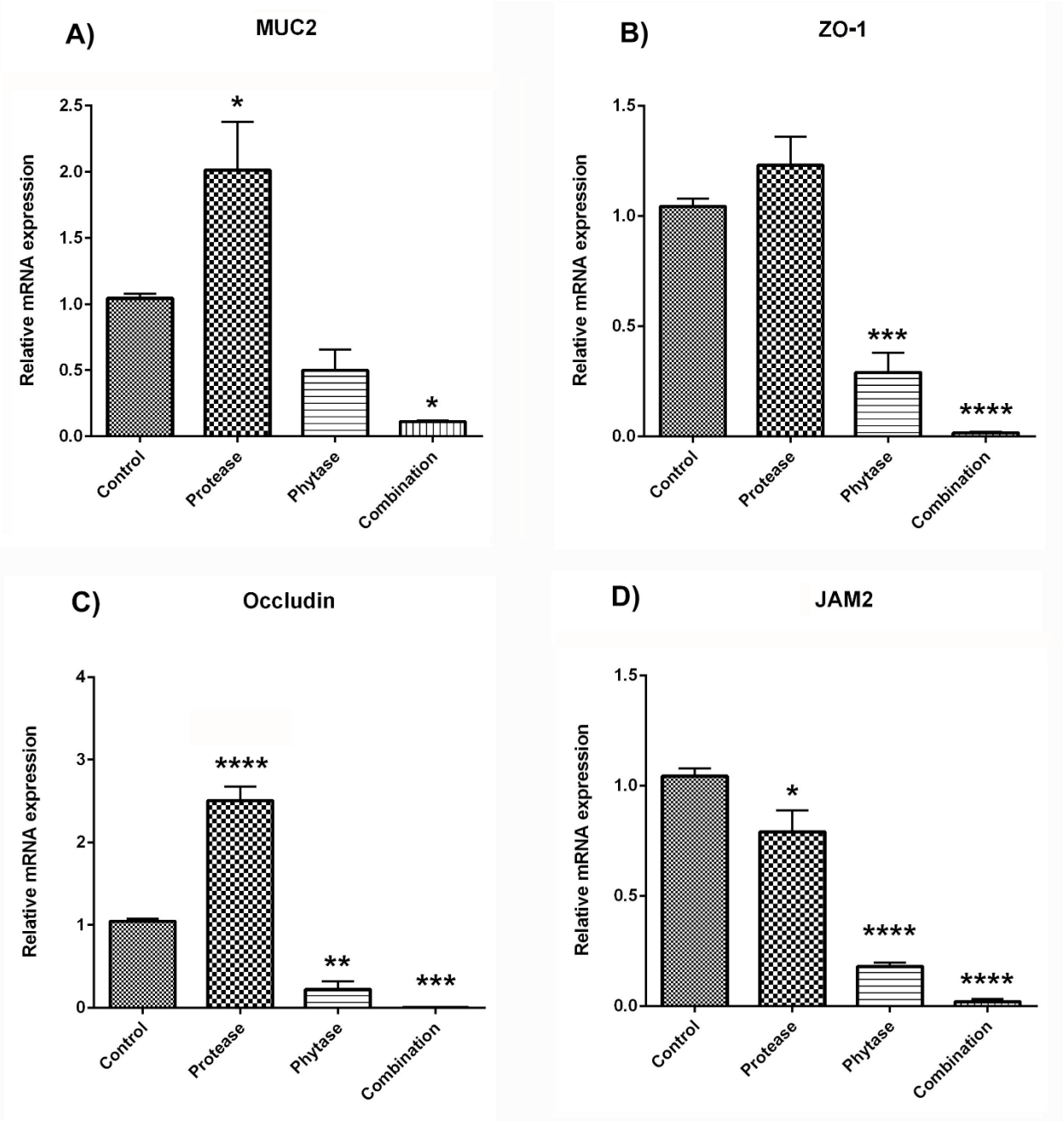

Figure 4: Effect of enzyme feed additives on the expression of tight junction protein genes in the ileum of broiler chickens. Panels A–D show the expression of MUC2, ZO-1, Occludin, and JAM2 genes in the ileum by real-time qPCR. Data are presented as mean with SEM (N = 3). Superscript asterisks on the bars indicate significant differences in comparing treatment to the control group (*, p<0.05; **, p<0.01, ***, p<0.001; ****, p<0.0001).

The physical barrier is a layer of epithelial cells connected by tight junctions (TJs), which maintain the integrity of the cell layer and play an essential role in regulating paracellular transport (Zihni et al., 2016). In the present study, the Occludin gene expression was 2.5 times higher in the group of chickens that received protease as a feed additive compared to the control group (p<0.0001) (Figure 4, C). For the other groups, a decrease in Occludin expression

was observed (p<0.01 for III and p<0.001 for IV groups). Occludin directly connects two cells, which makes it a key component of tight junctions (Raleigh, 2010).

The results obtained for Occludin may indicate better formation of tight junctions in the ileal epithelium of chickens receiving protease as a feed additive. In the case of ZO-1 gene expression, no significant differences were observed in the group receiving protease in the diet (p>0.05). At the same time, it was reduced in experimental groups III (p<0.001) and IV (p<0.0001) (Figure 4, B). The average expression levels of Occludin and ZO-1 genes are essential for forming tight gap junctions and, therefore, for intestinal health (Raleigh, 2010). A decrease in the expression of these genes may indicate a negative effect of phytase on the development of chickens.

One of the signs of a decrease in the inflammatory response is a decreased expression of the JAM2 gene. Junctional adhesion molecule 2 (JAM2) belongs to the immunoglobulin subfamily that forms tight junctions (TJ) in endothelial and epithelial cells. The level of JAM2 mRNA expression shows changes in the cells lining the vasculature, which may be necessary for assessing the effect of feed additives on the vascular system (Wong and Kinstler, 2023). JAM2 expression decreased for the group consuming feed with protease compared to the control group (p<0.05), which was also observed for the groups with the addition of phytase (p<0.0001) and with the combined additive (p<0.0001) (Figure 4, D). Compared to the control group, the decrease in JAM2 gene expression in the experimental groups indicates improved broiler health since increased JAM2 expression may indicate the presence of an inflammatory response (Johnson-Léger et al., 2002).

Conclusions

As a result of the study, a positive effect of subtilisin-like protease *B. pumilus* 7P (10 U/kg) and histidine acid phytase *P. brenneri* 3.5.1 (1000 FTU/kg) on poultry growth performance, digestibility, and assimilation of feed nutrients on Ross 308 broiler chickens has been observed. The present study revealed that protease and phytase do not affect the weight of the liver and lymphoid organs of chickens at 35 days old. Protease and phytase as a feed additive reduced inflammation in the ileum of chickens at 14 days old. Moreover, protease supplementation showed increased mucin gene expression and tight junction proteins, indicating an improvement in ileum. On the contrary, a decrease in the expression of these genes was detected in the case of phytase, which may indicate the need for further testing of the effect of phytase on poultry health. In conclusion, our data suggest that enzymes have a moderate effect on the function of the intestinal mucosa in chickens.

Article Information

Conflicts of Interest. The authors declare no conflict of interest.

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Authors contribution. DP and MS organized an experimental plan. ML and GL conducted experiments with birds and collected samples. DP and DK conducted experiments, analyzed experimental data, and prepared a manuscript. MS edited and approved the submitted manuscript.

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