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Effect of Ginger Derived Phyto-Protease on Production Performance, Serum Biochemistry, Nutrient Digestibility, Gut Morphometry and Immunity of Broilers Fed High Level of Poultry By Product Meal-Based Diet

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ABSTRACT

An experiment was conducted to assess the potential of ginger-derived phyto-protease on production performance, gut health, immunity, and nutrient digestibility of broilers fed a high level of poultry by-product meal-based (PBM) diet. A total of 320-day-old broiler birds were assigned to four dietary treatments CON, N-CON, GPP1, and GPP2. CON a commercial corn-soybean meal-based diet as per ROSS-308 nutritional specifications. N-CON was a 6.5% PBM-based diet and to this group, a ginger-derived phyto-protease was added at two levels in treatments GPP1 (50 mg kg^{-1} feed) and GPP2 (100 mg kg^{-1} feed), respectively. Birds in group N-CON performed poorly for the performance traits like feed intake, weight gain and FCR. Phyto-protease addition at 100mg kg^{-1} feed exhibited significant effect on body weight, FCR, and carcass traits compared to the control. The highest body weight gain was recorded in birds fed GPP2, followed by birds on GPP1 and the lowest in N-CON fed birds. Ginger-derived phyto-protease supplementation significantly lowered serum cholesterol (TG and LDL) while HDL was raised in GPP2 compared to the N-CON group showing a positive effect on lipid profile. Protein digestibility and AME were improved in the GPP2 group compared to N-CON. Gut health was improved in terms of better integrity, villus height, crypt depth, and villus surface area by birds in the GPP2 group. These findings demonstrated that adverse effects associated with using a high level of locally available animal protein concentrates could be ameliorated by supplementing birds with a ginger phyto-protease enzyme.

INTRODUCTION

The feed efficiency of poultry needs to be improved significantly because feed is the commodity with the highest cost about 70% of the total production (Deek *et al*., 2020). Poultry production at the commercial level is facing a big challenge regarding quality feed availability at stable prices on a regular basis. The poultry industry relies on soybean meal as the primary source of protein which fulfills up to 77% of its protein requirement (Ogunji, 2004). Many countries including Pakistan that imports full-fat soybean or meal face high price fluctuations and supply problems most of the time. According to USDA (2021) report, Pakistan imports 2.4 MMT of soybean costing 842 million US$ annually. Therefore, alternative indigenously available cheap protein sources will not only add versatility to feed ingredients for poultry but will also minimize soybean dependency to a certain extet (Shuaib *et al*., 2022). A locally manufactured poultry by-product meal (PBM) can potentially replace the plant protein source. PBM meal is produced by processing the inedible parts like viscera, feet, feathers, and heads of poultry carcass (Senkoylu *et al*., 2005) and is used as a protein source in the mono-gastric animal diet in many countries. Approximately 37% of the live weight of a broiler is considered slaughter house waste (Meeker and Hamilton, 2006) while non-edible parts account for approximately 28% of the live weight percentage of a broiler. In Pakistan, since there were 1407 million broilers in 2021 (Pakistan Economic Survey, 2021) with an average weight of 2kg and 28% non-edible parts they would produce

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787.92 million kg of waste product annually which needs to be managed efficiently to ensure environmental safety and reduce pollution. The use of this waste product as PBM in poultry ration will not only minimize this waste burden but will also minimize the cost of soybean meal and will act as a partial replacement for soybean. The CP value of PBM ranges from 45-65% (Karapanagiotidis *et al*., 2019) but aside from its benefits, there are some barriers to the incorporation of poultry by-product meal in broilers' diets which are its poor digestibility, quality of raw materials, processing conditions, variation in nutritive composition and inappropriate inclusion levels in poultry feed (Jafari *et al*., 2022).

Several researchers suggest different levels of PBM replacement with soybean meal, some suggest a 10% inclusion level however, Hassanabadi *et al*. (2008) reported a 5% inclusion level without adverse effects on broilers' performance while Jafari *et al*. (2022) reported reduced feed efficiency and growth rate beyond 5% inclusion level. To overcome these inherent problems of poultry by-product meal and make it suitable for use in broilers' diets several processing methods like fermentation or bio-procession, rendering, and enzymatic valorization can be effectively applied (Barnes *et al*., 2015; Lewis *et al*., 2019).

Enzymatic valorization with exogenous protease extracted from ginger might improve the feeding value of PBM by complementing the endogenous enzyme system, improving the breakdown of certain proteins, and enhancing digestibility (Classen *et al*., 1991). These inconsistent results about PBM inclusion level in broiler diets urge further studies to figure out the correct inclusion level without compromising broilers' health. Therefore, this study was conducted to investigate the effect of soybean meal replacement by 6.5% PBM on broilers' performance, nutrient digestibility, serum biochemistry and gut morphology of broilers supplemented with ginger derived phyto-protease enzyme at different concentrations.

MATERIALS AND METHODS

Experimental layout and bird's husbandry

This study was approved by the ethical committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan and was conducted in the poultry farm of the university. A total of 320-day-old broilers (ROSS-308) were purchased from a local hatchery and randomly distributed in four treatment groups in a completely randomized design. Each group had 4-replicates with 20 birds per replicate. Birds in all the treatment groups were presented with starter (0-21 days) and finisher (22-35 days) diets Table I as per

Table I. Experimental diets composition.

Ingredients	Starter phase (day1-21)		Finisher phase (day 22-35)	
	CON	N-CON	CON	N-CON
Corn	57.2	63.73	64.98	71.45
Soybean meal 44%	35.4	26.1	28.1	18.7
Poultry by-product meal	--	6.50	--	6.50
Animal fat	3.57	1.37	3.58	1.43
DCP	1.51	0.34	1.26	0.09
Limestone	1.10	0.63	1.01	0.55
DL-methionine	0.25	0.25	0.19	0.18
L-lysine HCL	0.07	0.25	0.53	0.22
L-threonine	0.02	0.06	0.01	0.05
NaCl	0.40	0.40	0.40	0.40
Vitamin premix[1]	0.25	0.25	0.25	0.25
Mineral premix[2]	0.09	0.09	0.09	0.09
Calculated nutrients %				
DM	87.4	87.4	87.4	87.2
M.E (Kcal/kg)	3100	3100	3180	3180
Crude protein	21.5	21.5	18.5	18.5
True protein	19.0	19.15	16.74	16.44
E.E	4.67	4.44	6.07	4.69
C.F	2.17	2.19	2.08	2.12
Ca	0.87	0.87	0.76	0.76
Total P	0.62	0.66	0.62	0.58
Avail. P	0.51	0.44	0.38	0.38
Na	0.22	0.21	0.18	0.21
Cl	0.27	0.31	0.27	0.31
Choline	1.85	2.05	1.74	1.93
Folate	0.86	0.87	0.82	0.83
D LYS	1.15	1.15	0.95	0.95
D MET	0.57	0.55	0.48	0.45
D TSAA	0.87	0.87	0.74	0.74
D THR	0.77	0.77	0.65	0.65
D TRP	0.25	0.22	0.21	0.18
D ARG	1.37	1.34	1.18	1.12
D VAL	1.07	1.02	0.92	0.88

[1]1kg of premix provided Vitamin E 80 mg, vitamin D3 3,000 IU, A 15,000 IU, vitamin B1 3 mg, vitamin K_3 3 mg, vitamin B_2 8 mg, vitamin B_3 60 mg, vitamin B_5 15 mg, vitamin B12 0.02 mg, Choline chloride (60%) 700 mg, vitamin B_6 4 mg, vitamin B9 2 mg, vitamin H 0.2 mg/Kg.
[2]Zn (as ZnSO4.H2O) 80 mg, Mn (MnSO4.H2O) 80 mg, Cu (CuSO4. H2O) 10 mg, Se 0.2 mg, Fe (FeSO4.H2O) 60 mg, I (KI) 1 mg.
CON, control; N-CON, 6.5% PBM-based diet; DCP, di calcium phosphate; DM, dry matter; ME, mobilizable energy; EE, ether extract; CF, crude fiber; LYS, lysine; MET, methionine; TSAA, total sulphure amino acid; THR, Threonine; TRP, TRP, tryptophan; ARG, arginine; VAL, valine

(ROSS-308) requirements using Brill Formulation® software. At different stages of rearing optimum environmental conditions of temperature, humidity, ventilation, and light were maintained as required. Ginger was purchased from the local market, washed, and sliced into small pieces. 200mL potassium phosphate buffer 100mM (pH 7.0) and 100g ginger were homogenized, then filtered through cheesecloth and centrifuged at 10,500x for 30 min. The supernatant was collected and used as a ginger protease (GP) with some modifications as described by Nafi *et al*. (2013). There were four dietary treatments (CON group; N-CON group, GPP1 group and GPP2 group). CON group was a normal corn-soybean meal diet, N-CON was a diet in which soybean meal was partially replaced with 6.5% PBM, GPP1 and GPP2 were the same 6.5%PBM (N-CON) based diets but added with ginger derived Phyto-protease enzyme at 50mg/kg and 100mg/kg.

Performance parameters

Feed intake was calculated by the formula as feed offered minus feed rejected. The final bird weight was subtracted from the initial weight to determine body weight gain. For each experimental unit, the feed conversion ratio (FCR) was calculated on weekly basis by dividing the feed consumed by the body weight of the birds.

To obtain dressing percentage carcass weight was divided by live weight (Badar *et al*., 2021). The relative weight of lymphoid organs (spleen, bursa, thymus) was calculated as organs weight as percent of BW= weight of an organ in g/ body weight in g × 100.

Serum biochemistry

Three birds per replicate were randomly selected for blood collection from wing veins in EDTA tubes. To separate serum, the samples were centrifuged at 3000 RPM for 15 min and then stored at -20°C till further analysis. Serum was analyzed by using commercial kits through standard procedures and protocols after thawing for determination of total cholesterol, high density lipoprotein (HDL), low density lipoprotein (LDL), and serum triglycerides (Asghar *et al*., 2021).

Hemagglutination inhibition (HI)

In this trial, birds were vaccinated through drinking water against New Castle Disease Virus (NDV) on days 7th and 17th. On day 7th birds were vaccinated against infectious bronchitis (IB). To determine antibody titer against NDV and IB three birds per replicate were randomly slaughtered for blood collection on day 35. A commercially available laboratory kit for HI was used for the determination of NDV antibody titer as described by Asghar *et al*. (2021). Antibody titer against IB was determined through ELISA.

Intestinal histomorphology

To evaluate intestinal histomorphology, 3cm duodenal tissue samples were taken from the duodenum center of three birds per replicate on day 35th. The digesta was gently removed and the tissue was flushed with normal saline and then preserved in buffer formalin (10%) for 48 h. In the histopathology lab, the tissue samples were washed with tap water and then treated with an alcohol solution. Using cassettes, a properly cut tissue section was embedded in paraffin wax. Microtome was used to cut tissue sections into 4µm, then mounting those tissue sections on slides and staining with hematoxylin and eosin (H and E). Under a computer-aided light microscope using image analysis (Leica Camera AG, Solms, Germany) histological indices were analyzed as described by Laudadio *et al*. (2012).

Nutrients digestibility

Five birds per replicate were shifted to metabolic cages on day 36 for total fecal collection for the last four days till day 42. A weighed amount of feed was offered daily and feces were collected early the next morning for four days and weighed. A representative sample (20%) of feces was placed in plastic bags and stored at -20°C till further analysis. For the determination of ileal nutrient digestibility 0.2% Cr_2O_3 was used as an indigestible marker. The fecal samples within the pen were pooled and dried in an oven at 65°C. The samples were ground to pass through a 0.5mm sieve. Similarly, digesta from the ileum portion of the small intestine of all the birds within the cage were collected and then frozen immediately. After drying, the samples were ground to pass through a 0.5mm sieve. The samples of diet, excreta and ileal digesta were analyzed for proximate analysis as described by AOAC (2005), and for calcium and phosphorus analysis by AOAC (2000). The concentration of chromium was determined with a UV absorption spectrophotometer (Hitachi Z-5000 Automatic Absorption Spectrophotometer, Tokyo, Japan) using the method of Pan *et al*. (2016). Adiabatic bomb calorimeter (Par instrument USA) was used to determine the gross energy of feed and fecal samples and then AME and nutrient digestibility were calculated using the formula of Stefanello *et al*. (2020) shown below.

$$\text{AME(IDE)(kcal/kg)} = \left\{ GE_i - \left(1 + GE_0 \times \frac{C_i}{C_0} \right) \right\}$$

Where GEi is gross energy (kcal/kg) of diet; GEo is gross energy (kcal/kg) of excreta. Ci and Co are concentrations of marker in the diet and digesta or excreta.

$$\text{Nutrient Digestibility (\%)} = \left\{ 1 - \left(\frac{C_i}{C_0} \times \frac{N_0}{N_i} \right) \right\} \times 100$$

Where; Ci and Co are concentrations of marker in the diet and digesta or excreta (%), respectively; Ni and No are

concentrations of nutrient in the diet and digesta or excreta (%), respectively.

Economics of the study (cost analysis)

Per chick cost was calculated for all the groups. The market rate of live birds per kg was used to calculate the mean gross return per chick.

$$\text{Feed cost/kg live weight} = \frac{\text{Per bird feed cost}}{\text{Live weight of bird}}$$

Statistical analysis

The collected data was analyzed by the standard procedure of (ANOVA) analysis of variance using a completely randomized design (CRD) as suggested by Steel and Torrie (1981) and the Tukey test was used for significance among means with a level of significance set at ($P<0.05$).

RESULTS

Growth performance

The growth performance results of the birds are shown in Table II. Feed intake was significantly ($P>0.05$) lower in the N-CON group in both starter (1-21 day) and finisher phases (21-35 day), while the feed consumption of the same N-CON feed supplemented with ginger-derived Phyto-protease enzyme in GPP1 and GPP2 groups was comparable with CON group. Ginger derived Phyto-protease enzyme supplementation significantly improved the body weight gain of broilers in both phases. Feed efficiency was improved ($P<0.05$) by ginger protease enzyme in GPP1 and GPP2 groups compared to CON and N-CON groups.

Carcass traits, lymphoid organs weight, and serum biochemistry

Results related to carcass traits, lymphoid organs weight, and serum biochemistry are shown in Table III. The carcass yield of broilers was significantly affected by ginger protease enzyme in GPP1 and GPP2 groups compared to N-CON. Carcass yield was highest in GPP2 and lowest in the N-CON group. Ginger derived phyto-protease enzyme significantly affected dressing percentage. The dressing percentage of N-CON was the lowest and GPP2 was the highest. The percent relative weight of lymphoid organs (spleen and bursa) was significantly affected by ginger protease. Maximum weight of the spleen and bursa was noticed in the GPP2 group and minimum in the N-CON group. No significant effect of ginger protease was observed on the thymus. Total cholesterol, LDL, and triglycerides were ($P<0.05$) lowered, and HDL levels were raised in ginger protease-supplemented groups compared to control and negative control groups, the maximum effect of ginger protease was observed in the GPP2 group. The lowest values of total cholesterol, LDL, and triglycerides were observed in ginger protease-supplemented groups and highest in the N-CON group. HDL level was improved and highest in the GPP2 group and lowest in the N-CON group.

Table II. Effect of different levels of a ginger derived phyto-protease enzyme on broiler bird's performance fed on PBM based diet on day 21 and 35.

Parameters	CON	N-CON	GPP1	GPP2	P value
Starter phase					
Initial weight (g)	39.7±0.54	39.2±0.55	39.0±0.49	40.6±0.58	0.192
BWG (g)	704.2±0.62^{a}	679.7±1.05^{b}	713.2±0.62^{a}	719.7±1.75^{a}	0.001
Feed intake (g)	1112±0.40^{a}	1076±1.24^{b}	1114±2.10^{a}	1121±2.25^{a}	0.003
FCR	1.57±0.50^{a}	1.58±0.04^{a}	1.56±0.08^{ab}	1.55±0.75^{b}	0.001
Finisher phase					
BWG (g)	1001±1.50^{ab}	955±2.30^{b}	1028±2.33^{a}	1042±1.87^{a}	0.009
Feed intake (g)	2193±0.40^{a}	2116±2.74^{b}	2195±0.64^{a}	2204±0.85^{a}	0.002
FCR	2.19±0.50^{a}	2.21±0.02^{a}	2.13±0.08^{b}	2.11±0.08^{b}	0.002
Overall period					
Final weight (g)	1745±1.46^{b}	1674±1.93^{c}	1780±2.60^{ab}	1803±3.40^{a}	0.002
BWG (g)	1705±1.46^{b}	1634±1.93^{c}	1741±2.60^{ab}	1762±3.40^{a}	0.001
Feed intake (g)	3305±0.70^{a}	3192±3.61^{b}	3310±1.82^{a}	3326±1.82^{a}	0.008
FCR	1.89±0.50^{a}	1.91±0.01^{a}	1.85±0.88^{b}	1.84±0.50^{b}	0.002

CON, Normal commercial ration; N-CON, 6.5% of PBM inclusion; GPP1, 6.5%PBM +50mgkg^{-1} phyto-protease; GPP2, 6.5% PBM+100mgkg^{-1} phyto-protease. Means within the same rows bearing different superscripts differ significantly ($p \leq 0.05$), FI, Feed intake; BWG, body weight gain; FCR, Feed conversion ratio.

Table III. Effect of different levels of a ginger derived phyto-protease (GPP) enzyme on carcass traits and serum biochemistry of broiler birds fed on PBM based diets on day 35.

Parameters	CON	N-CON	GPP1	GPP2	P-value
Dressing percentage (%)	$62.3^{bc}\pm0.20$	$61.2^{c}\pm2.00$	$64.4^{ab}\pm0.17$	$65.3^{a}\pm0.09$	0.049
Spleen (%)	$0.12^{b}\pm0.88$	$0.10^{b}\pm0.78$	$0.13^{ab}\pm0.88$	$0.16^{a}\pm0.50$	0.001
Bursa (%)	$0.12^{ab}\pm0.45$	$0.10^{b}\pm0.01$	$0.12^{ab}\pm0.45$	$0.15^{a}\pm0.45$	0.004
Thymus (%)	0.36±0.08	0.36±0.01	0.35±0.78	0.35±0.64	0.297
Cholesterol (mg/dL)	$149.9^{ab}\pm0.51$	$151.7^{a}\pm0.71$	$137.8^{ab}\pm0.61$	$134.7^{b}\pm1.01$	0.013
Triglycerides (mg/dL)	$46.5^{a}\pm0.55$	$40.5^{ab}\pm0.34$	$26.5^{c}\pm0.34$	$31.0^{bc}\pm0.25$	0.001
HDL (mg/dL)	$63.8^{b}\pm0.53$	$64.2^{b}\pm0.72$	$71.6^{ab}\pm0.29$	$78.5^{a}\pm0.49$	0.004
LDL (mg/dL)	$71.1^{a}\pm0.23$	$70.9^{a}\pm0.60$	$60.4^{ab}\pm0.53$	$49.6^{b}\pm0.23$	0.008

For statistical details and details of treatments groups, see Table II. HDL, high density lipoprotein; LDL, low density lipoprotein.

Table IV. Effect of different levels of a ginger derived phyto-protease enzyme on immune response and intestine (duodenum) histomorphology of broilers fed on PBM based diets on day 35.

Parameters	CON	N-CON	GPP1	GPP2	P-value
ND (log^2)	$4.22^{b}\pm0.12$	$4.17^{b}\pm0.14$	$5.15^{a}\pm0.17$	$4.88^{a}\pm0.08$	0.005
IB (log^2)	$4.95^{b}\pm0.06$	$4.92^{b}\pm0.13$	$4.97^{b}\pm0.08$	$5.42^{a}\pm0.08$	0.008
Livability (%)	$90.0^{ab}\pm0.00$	$85.0^{b}\pm2.88$	$95.0^{ab}\pm2.88$	$97.5^{a}\pm2.50$	0.014
Villi height (µm)	$730.9^{c}\pm0.47$	$711.9^{d}\pm0.60$	$897.1^{b}\pm0.45$	$902.3^{a}\pm0.68$	0.002
Villi width (µm)	$50.8^{c}\pm0.74$	$43.7^{d}\pm0.59$	$55.4^{b}\pm0.76$	$60.4^{a}\pm1.22$	0.001
Crypt depth (µm)	$82.5^{c}\pm0.97$	$62.2^{d}\pm0.79$	$90.2^{b}\pm0.77$	$115.9^{a}\pm0.87$	0.002
VSA (mm)	$0.058^{c}\pm0.71$	$0.048^{d}\pm0.77$	$0.078^{b}\pm0.74$	$0.085^{a}\pm0.70$	0.003

For statistical details and details of treatments groups, see Table II. ND, new castle disease; IB, infectious bronchitis; VSA, villus surface area.

Immunity and gut morphometry

The effect of soybean meal replacement with PBM at a 6.5% level added with or without ginger derived phyto-protease on broilers' immune response, livability, and gut morphology is shown in Table IV. On day 35th the antibody titer against NDV was significant and highest in the GPP1 group while lowest in the N-CON group. The livability of broilers was significantly high in the GPP2 group compared to other treatment groups. The highest livability was observed in the GPP2 group and the lowest in the N-CON group. The antibody titer against infectious bronchitis was significantly affected by ginger protease supplementation. The highest titer was observed for the GPP2 group and the lowest in the N-CON group.

Gut morphometry of broilers was significantly affected and the GPP2 group observed significantly the highest villi length, width, and crypt depth compared to all other groups while the N-CON group showed the lowest villi length, width, and crypt depth. Villus surface area was significantly high in the GPP2 group compared to the N-CON group.

Nutrients digestibility

Results regarding nutrient digestibility and economics of broilers supplemented with ginger-protease enzyme are shown in Table V. Mean percent digestibility of dry matter, crude protein, crude fiber, calcium, and phosphorus improved significantly high in the GPP2 group and lowest in the N-CON group. The AME was significantly higher in the CON group followed by GPP2, and GPP1, and lowest in the N-CON group.

Economics

The results of economic parameters indicated that feed cost per chick was significantly low in the N-CON group but the gross return was significantly high in GPP2 and GPP1 groups compared to CON and N-CON groups. Despite the same feed cost GPP2 gross return and profit over feed cost were higher than the CON group.

Table V. Effect of different levels of a ginger derived phyto-protease enzyme on nutrients digestibility and economics of broiler birds fed on PBM based diets on day 42.

Parameters	CON	N-CON	GPP1	GPP2	P-value
DM (%)	$57.2^{b}\pm0.12$	$57.5^{b}\pm1.89$	$60.6^{ab}\pm0.18$	$61.7^{a}\pm0.01$	0.012
CP (%)	$41.5^{c}\pm0.16$	$41.5^{c}\pm0.18$	$45.1^{b}\pm0.07$	$46.1^{a}\pm0.02$	0.024
CF (%)	$18.6^{ab}\pm0.22$	$18.4^{b}\pm0.28$	$19.1^{ab}\pm0.09$	$20.6^{a}\pm0.19$	0.045
Ca (%)	$40.0^{ab}\pm0.64$	$39.4^{b}\pm1.28$	$41.0^{ab}\pm0.64$	$42.1^{a}\pm0.11$	0.049
P (%)	$38.7^{bc}\pm0.26$	$37.5^{c}\pm0.84$	$40.1^{ab}\pm0.42$	$41.0^{a}\pm0.39$	0.002
AME (Kcal/Kg)	$2995^{a}\pm0.64$	$2894^{b}\pm4.20$	$2934^{ab}\pm1.08$	$2943^{ab}\pm0.85$	0.035
Feed cost (PKR/Kg)	70	60	66	70	N/A
Feed cost/kg live weight	$132.5^{a}\pm0.26$	$114.4^{c}\pm0.47$	$122.7^{b}\pm0.22$	$129.1^{ab}\pm0.19$	0.002
Gross return (PKR)	$349.^{ab}\pm0.72$	$334.8^{b}\pm0.99$	$356.0^{ab}\pm0.68$	$360.6^{a}\pm0.72$	0.027
Profit over feed cost	216.4±0.98	220.4±1.33	233.3±0.92	231.5±0.91	0.109

For statistical details and details of treatments groups, see Table II. DM, dry matter; CP, crude protein; CF, crude fiber; Ca, Calcium; P, phosphorus; AME, apparent metabolizable energy. Values are given in Pak-rupees (1US$=175).

DISCUSSION

This study was conducted to assess the effect of soybean meal replacement with poultry by-product meal at a 6.5% level of incorporation on broiler performance supplemented with or without ginger-protease enzyme. Feed intake, weight gain (WG), and FCR were improved with ginger protease supplementation in PBM-based diets. Depression in the growth performance of birds was obvious when fed PBM-based diets only, however with the supplementation of ginger-protease enzyme the growth performance improved significantly, this indicated that ginger-protease was responsible for complementing the endogenous secretion of proteolytic enzymes resulting in improved performance. The overall performance of birds was improved by enzyme supplementation which agrees with the previous findings of Cowieson *et al*. (2006), Mahmood *et al*. (2018), and Saaci *et al*. (2018) who noticed a positive correlation of aqueous ginger extract on body weight gain and feed intake of broilers. Mahmood *et al*. (2018) also reported improved feed intake with protease enzyme in PBM-based diets. Similarly, a positive effect of protease enzyme was noticed on carcass traits of broilers by Mahmood *et al*. (2018), while, Barazesh *et al*. (2013) reported that carcass characteristics were not affected by different concentrations of ginger opposing our observations. Similarly, broilers provided with protease in low-protein diets showed improvement in carcass yield (Ajayi, 2015). Enhanced utilization and deposition of protein might be responsible for improved carcass yield in phyto-protease-supplemented groups. Improvement in the percent weight of bursa and spleen was observed for ginger protease-supplemented groups (Raeesi *et al*., 2010) approving our observations. The per cent weight of the thymus was not affected by dietary ginger protease supplementation (Bondona *et al*., 2019) and these results agreed with our findings of a non-significant effect on thymus per cent weight. The non-significant effect of ginger protease on thymus weight was also confirmed by other researchers (Khushdil *et al*., 2012).

Serum cholesterol, triglycerides, and LDL level was significantly lowered while HDL level was improved in ginger protease-supplemented groups compared to CON and N-CON groups. Reduction in cholesterol and triglycerides were reported by feeding 0.2% ginger to broilers (Mohamed *et al*., 2012). The presence of HMG-COA (hydroxyl methyl glutaryl coenzyme A reductase) in ginger which is the rate-limiting step in cholesterol synthesis might be responsible for its hypolipidemic effect (Asghar *et al*., 2021). Cholesterol-lowering effects of the aqueous ginger extract were previously reported by Oleforuh *et al*. (2015). The antibody titer against Newcastle disease was significantly high among ginger derived phyto-protease supplemented groups on the 35th day. Deniz Azhir *et al*. (2012) also reported the immune-boosting effect of ginger on broilers' immune systems. The increase in amino acid digestion by ginger protease might be responsible for the increase in antibody titer (Li *et al*., 2007) as dietary protein deficiency has long been known to impair immune functions and make humans and animals more susceptible to infectious diseases. The antioxidant effect of ginger and the presence of natural aromatic compounds like shogoals and gingerol might be another possible reason for the immune boosting effect of ginger

protease (Khan *et al*., 2012).

Dry matter, crude protein, crude fiber, calcium, and phosphorus were significantly affected by ginger-protease enzyme in PBM-based diets. Our results were consistent with the observations of Kamel *et al*. (2015) and Duwa *et al*. (2020). However, Saaci *et al*. (2018) did not detect any significant effect of ginger extract on the DM and CP digestibility of broilers. This may be due to different types of feed, the duration of the experiment, and crude ginger extract. Digestibility of CP was improved by protease supplementation in broilers' diet (Vieira *et al*., 2013). The AME was enhanced in ginger protease-supplemented groups which may be attributed to the extra-proteinaceous effect of protease (Cowieson and Roos, 2014) that aid in the digestibility of fats apart from proteins thus improving AME. The significant changes among treatment groups for nutrient digestibility may be attributed to ginger derived phyto-protease enzymes contributing positively to digestive functions by stimulating the endogenous enzyme system. Broiler health and performance are affected by the development of the small intestine which is an important digestive organ involved in nutrient absorption (Kawalilak *et al*., 2010). To evaluate the effects of nutrients on gastrointestinal physiology a common measurement tool is villus structure and anatomy. Moreover, crypt depth and villus height have been documented to show significant correlations regarding performance improvement. By increasing the crypt depth and absorptive surface area of the small intestine of broilers, ginger protease improved the digestive and absorptive capacity which resulted in faster growth rates (Prakash and Srinivasan, 2012). The reduction in villus length, width, crypt depth, and surface area may be due to poor absorption of nutrients in PBM-based diets, however, the gut morphometry was significantly affected and villi length, villi width, and crypt depth were significantly improved by ginger derived phyto-protease supplementation in a PBM-based diet. Our results agreed with the reports of Karangiya *et al*. (2016), Shewita and Taha (2018) and Asghar *et al*. (2021) that there were improvements in the villus height, width, and surface area of the birds' duodenum. This increase in villi length and width causes an increase in the surface area of villi increasing the absorption of nutrients and resulting in improved growth and performance (Oladele *et al*., 2012). The electrolytes are produced through the excretory activity of crypt cells into the intestinal lumen ultimately boosting up digestive activities of the gut (Bowen, 2011).

CONCLUSION

The results of the current study clearly indicated that the negative effects of PBM on broilers' growth performance can be successfully alleviated through the addition of ginger derived Phyto-protease. This significantly improved broilers' performance, gut health, immunity, and nutrient digestibility in PBM-based diets. PBM may be used successfully as a partial replacement of soybean meal at 6.5% level of inclusion with the supplementation of ginger derived phyto-protease at (100 mg kg^{-1} feed) without any antagonistic effects on broilers health.

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IRB approval

The study was approved by Board of Advanced Studies and Research (BASR), (NO.332PS/UAP).

Ethical statement

This study was approved by the animal welfare and care committee of the Faculty of Animal Husbandry and Veterinary Sciences, The University of Agriculture, Peshawar, Pakistan, and all the measures and tools were considered to minimize the pain and discomfort of birds during the conduction of this experiment.

Statement of conflict of interest

The authors have declared no conflict of interest.

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