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Effects of Bromelain on Growth Performance, Biochemistry, Antioxidant Metabolism, Meat Quality, and Intestinal Morphology of Broilers

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HIGHLIGHTS

- Bromelain is a mix of proteolytic enzymes.
- Bromelain is considered a safe phytotherapeutic agent.
- Bromelain improves small intestine morphology.
- Bromelain at 30 g/kg diet dose improved final Broiler performance.

Abstract: Bromelain is a mix of proteolytic enzymes obtained from the pineapple plant's fruit or stem. The effect of various rates of bromelain supplementation on broiler growth and carcass performance, meat quality, antioxidant metabolism, and blood profiles were examined in this study. In total, 288 male broiler chicks (Ross 308) one-day-old were used to determine the effects of different doses of bromelain (0, 0.15, 0.30, and 0.45 g / kg diet) during the six-week trial period. The trial groups consisted of six replicates of twelve animals each. Bromelain (30g/kg diet) improved the feed conversion ratio (FCR) and increased final body weight (BW) and body weight gain (BWG) ($P<0.05$). Bromelain increased malondialdehyde (MDA) levels in the drumstick tissue ($P<0.05$). Bromelain decreased serum cholesterol (COL), High-density lipoprotein (HDL), and Low-density lipoprotein (LDL). Bromelain did not affect drumstick and breast meats' pH value but had shown a

limited and variable effect on the color parameters and Thiobarbituric acid reactive substances (TBARS) during the storage period. Bromelain increased goblet cell number (GCN), crypt depth (CD), villus length (VL), and epithelial height (EH) ($P<0.05$) in the small intestine. In conclusion, bromelain had a minor impact on meat microbiological quality but improved intestinal morphology and final performance parameters.

Keywords: bromelain; chicken; enzyme; meat quality.

INTRODUCTION

Bromelain is a proteolytic enzyme belonging to the cysteine peptidase family obtained from the pineapple plant's fruit (EC 3.4.22.33) or stem (EC 3.4.22.32) [1–4]. Bromelain is used in varied industries, including food, textiles, and pharmaceuticals [2]. Eight forms with proteolytic activity have been isolated from bromelain [5]. These enzymes have anti-edema, anti-inflammatory [6], anticancer [7], antioxidant [8], and anti-thrombotic and fibrinolytic activities [9]. Bromelain is considered a safe phytotherapeutic agent [10,11] and even high doses of bromelain prepared for clinical use are reported to be safe [12]. It is reported that bromelain can be used for long periods of daily dosages ranging from 200 to 2,000 mg/kg. [13]. Bromelain is quickly absorbed by the body and retains its function in the gastrointestinal tract [4]. The structure of the bromelain enzyme is resistant to stomach acid and can absorb up to %40 in the digestive tracts of murine [14] and remain intact and proteolytically active [15].

In poultry, the digestive system, which is anatomically shaped during the embryonic period, gains functionality with the effect of feeding and with age [16–18]. Exogenous proteases are used to complement the effect of enzymes in the animal's digestive system to increase the nutritional value of feeds and also to hydrolyze certain types of proteins resistant to endogenous secretory enzymes [19–22]. Bromelain, which is a proteolytic enzyme, may use as a supplement to improve food digestibility. Bromelain can be used effectively in the absence of pepsin and trypsin enzymes because it has a proteolytic effect and maintains its activity across a broad pH range [14,23]. Akit and coauthors [16] reported that dietary bromelain improved protein and fat digestibility resulting in reduced fecal nitrogen content, increased intestinal villus height, and reduced digesta viscosity in the broiler chickens. In another study, it was reported that bromelain decreases the amount of serum cholesterol, and increases serum antioxidant enzyme concentrations so bromelain could be added to the laying hen diet up to 0.45 g/kg to boost antioxidant capacity [24].

Although there are many studies on bromelain, there is limited research on its use as a feed supplement in broilers. The effects of varying amounts of bromelain as a feed supplement on broiler growth and carcass performance, meat quality, antioxidant metabolism, and blood profile were examined in this study.

MATERIAL AND METHODS

The study's experimental protocol was approved by the Atatürk University Animal Experiments Local Ethics Committee's decision dated 19.04.2016 and numbered 52.

The material of the study a total of 288 one-day-old broiler chicks (Ross 308) were constituted. Birds were divided into 4 groups with 6 replications and 12 animals in each repetition. All animals were fed the same starter (1-21 days) and finisher diets (22-42 days). Diets were formulated according to nutrient requirements defined by the National Research Council (NRC) for Broiler chickens [25]. The diet compositions are shown in Table 1. The experimental diets were a bromelain-free basal diet and the bromelain-added basal diet (from pineapple, 2000 GDU per g; Solgar Inc., Leonia, NJ) at 0.15, 0.30, and 0.45 g/kg concentrations. Throughout the study period, feed and water were available ad libitum. The environmental temperature was maintained at 32-35 °C in the first week, then progressively decreased to 22 °C until the end of the experiment. Artificial lighting was applied for 23 hours a day during the trial period. The dietary nutritional composition has been determined, according to AOAC [26].

Table 1. Ingredients and Chemical Composition of the Basal Diets, g/kg.

	Starter (1 to 21 d)	**Finisher (22 to 42 d)**
Ingredients g/kg		
Corn	514.6	562.0
Soybeanmeal (%44)	393.5	316.5
Soybean oil	31.8	66.0
Dicalcium phosphate	21.1	17.3

Cont. Table 1

Wheat bran	15.0	15.0
Calcium carbonate	9.6	8.6
Sodium bicarbonate	2.8	2.7
Salt	2.7	2.8
Vit-Min. Premix*	5	5
DL-methionine	2.4	2.6
L-lysine HCL	0.8	0.6
L-threonine	0.7	0.9
Calculated analysis		
Metabolizable energy, MJ/kg	12.22	13.40
Crude protein, %	22.03	19.02
Ether extract%	3.36	5.68
Crude fiber %	2.79	2.63
Lysine, %	1.18	1.03
Methionine, %	0.56	0.51
Calcium, %	0.93	0.80
Phosphorous, %	0.63	0.57

* Contents per kilogram: vitamin A, 3.600.000 U; vitamin D3, 800.000 U; vitamin E, 7200 U; vitamin K3, 800 mg; thiamine, 720 mg; riboflavin, 2640 mg; calcium pantothenate, 4000 mg; niacin, 12,000 mg; pyridoxine, 1200 mg; folic acid, 400 mg; vitamin B12, 6 mg; biotin, 40 mg; choline, 100,000 mg; Mn, 39680 mg; Fe, 20000 mg; Zn, 33880 mg; Cu, 4000 mg; I, 400 mg; Se, 80 mg.

The BW, BWG, and feed intake (FI) were measured weekly, and survivability was recorded daily. The FCR was calculated as the following formula: (FI (g) / BWG (g)). Two birds from each replication were chosen at 42 days of age, weighing an average of pen weight. They were slaughtered after determining their final body weight. After the sacrifice process, the feet were cut and head and viscera were removed and the carcasses were cleaned. After keeping the carcasses at + 4 °C for 24 hours, cold carcass yield was determined, by weighing [(cold carcass weight/slaughter weight) ×100].

Broilers, which were slaughtered and brought to the laboratory under hygienic conditions, and drumstick and breast meat were separated and stored at 4 ± 1 ℃ for 9 days. As chemical analysis on meat samples on the 1, 3, 5, 7, and 9th days of the storage; water activity (aw), pH, TBARS, color [L * (relative lightness), a * (relative redness), b * (relative yellowness)] analyzes were performed. Microbiological analyzes of the samples were performed according to the method described by Baumgart and coauthors [27]. 25 g of the meat mixture of a thigh and a breast sample were homogenized with 225 mL of sterilized Ringer's solution. Then, the other solutions were prepared. The spread-plate technique was used for inoculation. The number of TMAB was determined on the growth medium Plate Count Agar (PCA, Merck, Germany). Plates were incubated at 30 ± 1 ºC for 72+1 hours under aerobic conditions. The total number of TPAB was determined on the PCA growth medium. Plates were incubated at 7 ± 1 ºC for ten days under aerobic conditions. The number of *Lactobacillus spp.* was determined on De Man, Rogosa, and Sharpe agar (MRS, Merck, Germany). Plates were incubated at 37±1 °C for 72 hours under anaerobic conditions. The dilutions whose coliform counts were appropriate were inoculated in a volume of 0.1 ml in VRBA (Violet Red Bile Agar, Merck, Germany) growth medium. Petri plates were incubated for two days at 30 ºC under anaerobic conditions. The number of Micrococcus/Staphylococcus was determined on Mannitol Salt Agar (MSA, Merck, Germany) growth medium. Plates were incubated at 30±1 ºC for 48±1 hours under aerobic conditions. The number of *Pseudomonas spp*. was determined in Pseudomonas Agar Base (Oxoid, UK) growth medium with CFC (Cephalothin, Fucidin, Cetrimide). Plates were incubated at 30 ± 1 ºC for 48±1 hours under aerobic conditions. The yeast and mould number were determined Rose Bengal Chlorophenical Agar (RBC, Merck., Germnay) and plates were incubated 20±1 °C for 5 days under aerobic condition. The determined bacterial counts were expressed as log cfu g^{-1}. Aqualab 4TE (USA) device was used to determine the aw value. pH values were determined by pH meter (WTW Inolab, Germany) after homogenization [28]. TBARS were analyzed as described by Lemon [29] and results are given in µmol malonaldehyde/kg.

The cross-sectional surface color intensities (L *, a *, b *) of the samples were determined using the Minolta (CR-200, Minolta Co, Osaka, Japan) colorimeter. Analysis was performed three times for each sample.

The blood samples collected during the sacrifice process of the animals were centrifuged at 1500 rpm for 5 minutes, and the serum samples obtained were stored at -80 °C until biochemical analyses. In blood serum samples; Glucose, total protein, albumin, triglyceride, cholesterol, LDL, HDL levels were determined using commercial kits on the Beckman Coulter AU5800 (Beckman Coulter Inc., USA) auto analyzer.

For the measurement of enzyme activities, the tissue samples taken after the sacrification process were washed with PBS before they were homogenized with liquid nitrogen. Then, tissue samples were homogenized in Tissue Lyser (30 Hz 3 minutes) by adding 0.1 gr tissue sample + 1ml homogenate buffer.

In tissue samples homogenized with appropriate homogenate buffers;

MDA: Malondialdehyde TBARS Assay Kit, Item No: 10009055 (CAYMAN)

GSH: Glutathione Assay Kit, Item no: 703002 (Cayman)

SOD: Superoxide Dismutase Assay Kit Item No: 706002 (CAYMAN)

CAT: Catalase activity was measured by applying the rules stated by Aebi [30].

For histological examinations from sacrificed animals; Duodenum, jejunum, and ileum tissues were taken cut from the middle regions. Tissues were fixed in %10 buffered formol solution for 48 hours, then embedded in paraffin blocks by passing through alcohol and xylol series by routine histological methods. Sections taken from paraffin blocks with Leica RM2125RT microtome (Leica Microsystems, Wetzlar, Germany) with a thickness of 5 µm were stained with Crossman's triple staining to evaluate crypt depth, villus length, and epithelial height. Periodic acid Shift (PAS) dye was applied to 5-7 µm transversal serial sections from each block to determine the distribution and histochemical structure of the goblet cells. For this purpose, goblet cells in the villi and crypt epithelium of the region of 30000 µm (20 lenses) length were counted in the villi and crypts in serial sections taken from each block. The average of goblet cells falling to 1 mm was measured manually using the image analysis program (Kameram SLR 6.1; Mikro Sistem Ltd, Turkey) and their arithmetic averages were calculated.

The normal distribution of the data was checked by the Kolmogorov-Smirnov test. The distribution was found to be normal ($P>0.05$). The data were analyzed by one-way analysis of variance (ANOVA), and Duncan's test through IBM SPSS 20 for macOS. A P value of 0.05 was considered the limit of significance. Data are presented as the mean ± standard error (SE).

RESULTS

Growth performance data are shown in Table 2. Bromelain at a dietary dose of 30 g/kg significantly improved BW, BWG, and FCR at 6. weeks compared to the control group.

Table 2. Effects of dietary bromelain supplementations on the growth performance in broiler chickens.

	Groups				
Items	**CONT**	**BRO-15**	**BRO-30**	**BRO-45**	***p-values***
BW, (g)					
W 1	220.38±2.12	216.39±1.71	215.49±1.76	218.60±3.12	ns
W 2	544.54±1.33	536.59±6.93	534.41±5.08	548.92±5.79	ns
W 3	1039.08±16.20	1033.10±9.81	1037.51±15.78	1034.29±22.54	ns
W 4	1556.73±24.87	1640.90±17.75	1592.00±31.17	1616.57±38.06	ns
W 5	2267.83±42.33	2383.55±49.43	2335.27±53.93	2272.88±82.52	ns
W 6	2766.99±92.06^{a}	3060.21±101.28^{ab}	3106.93±98.14^{b}	2836.53±106.14^{ab}	*
BWG, (g)					
W 1	23.61±0.23	23.18±0.18	23.09±0.19	23.42±0.33	ns
W 2	46.31±0.24	45.74±0.78	45.56±0.49	47.19±0.43	ns
W 3	70.65±2.40	70.93±0.77	71.87±1.58	69.34±2.44	ns
W 4	73.95±1.95^{a}	86.83±1.65^{b}	79.21±2.42^{ab}	83.18±3.98^{b}	*
W 5	101.59±2.84	106.09±4.98	106.18±5.24	93.76±7.64	ns
W 6	71.17±10.77^{a}	96.67±8.98^{ab}	110.24±7.89^{b}	80.52±4.82^{a}	*
FI, g					

Cont. Table 2

W 1	24.58±0.64	23.58±0.58	23.03±0.65	23.79±0.54	ns
W 2	58.15±1.60	58.75±0.46	57.88±0.91	60.32±1.10	ns
W 3	105.23±1.81^{b}	98.02±1.26^{a}	94.15±0.76^{a}	98.89±2.02^{a}	*
W 4	112.27±1.58^{a}	125.17±1.68^{b}	125.68±1.91^{b}	126.84±1.63^{b}	*
W 5	174.03±0.89^{b}	174.51±5.41^{b}	176.16±4.17^{b}	160.60±3.39^{a}	*
W 6	132.56±6.20^{a}	169.74±10.77^{bc}	180.59±3.89^{c}	147.99±7.47^{ab}	*
FCR					
W 1	1.04±0.02	1.02±0.02	1.00±0.02	1.02±0.01	ns
W 2	1.26±0.03	1.29±0.02	1.27±0.01	1.28±0.02	ns
W 3	1.49±0.04^{c}	1.38±0.02^{ab}	1.31±0.02^{a}	1.43±0.05^{bc}	*
W 4	1.52±0.03^{ab}	1.44±0.02^{a}	1.59±0.03^{b}	1.54±0.06^{ab}	*
W 5	1.72±0.05	1.65±0.03	1.68±0.07	1.79±0.15	ns
W 6	2.14±0.24^{b}	1.78±0.05^{ab}	1.68±0.09^{a}	1.85±0.07^{ab}	*
Carcass yield (%)	67.08 ± 1.11	68.26 ± 0.80	71.298 ± 3.54	67.46 ± 0.76	ns

The values are given as mean ± SEM, (n=72 in each group). a, b, c: Means in the same row with different superscripts differ significantly (*: P<0.05). ns: not significant (P>0.05).

The levels of MDA were increased in the breast tissues in BRO 30 group (p<0.05) compared to the CONT group (Table 3).

Table 3. Effects of dietary bromelain supplementations on antioxidant metabolism in breast, and drumstick tissues of broiler chickens.

Items	**Groups**				
	CONT	**BRO-15**	**BRO-30**	**BRO-45**	***p-values***
CAT, *µmol/min/mg*					
Breast	195.49±22.21^{b}	257.28±16.45^{a}	160.38±8.76^{b}	192.18±25.31^{b}	*
Drumstick	176.40±23.98^{b}	258.51±23.03^{a}	240.12±28.67^{ab}	240.61±18.78^{ab}	*
SOD, *U/mg*					
Breast	50.59±0.68	44.83±3.76	47.58±2.90	53.90±3.66	ns
Drumstick	43.00±1.24^{b}	45.55±2.26^{b}	47.38±1.23^{ab}	51.06±1.76^{a}	*
GSH, *µmol/mg*					
Breast	35.98±3.49^{ab}	35.32±4.73^{ab}	45.88±1.88^{a}	32.56±3.15^{b}	*
Drumstick	46.92±9.92	43.81±1.17	44.80±3.00	45.93±1.75	ns
MDA, *µmol/mg*					
Breast	40.50±2.42^{b}	54.55±4.14^{ab}	66.78±6.95^{a}	47.73±4.33^{b}	*
Drumstick	35.23±1.79^{b}	51.05±2.97^{a}	50.32±4.21^{a}	52.05±2.63^{a}	*

The values are given as mean ± SEM. a, b, c: Means in the same row with different superscripts differ significantly (*: P<0.05). ns: not significant (P>0.05). CAT: catalase, SOD: superoxide dismutase, GSH: glutathione, MDA: malondialdehyde.

The levels of COL, HDL, and LDL significantly decreased (P<0.05) in all bromelain treatments groups compared with the CONT group (Table 4).

Table 4. Effects of dietary bromelain supplementations on serum parameters.

Items	**Groups**				
	CONT	**BRO-15**	**BRO-30**	**BRO-45**	***p-values***
ALP (U/L)	2243.33±237.91	2597.67±682.65	2079.83±313.70	1700.67±289.34	ns
TG (mg/dL)	31.00±2.10^{b}	40.50±3.52^{ab}	47.17±5.44^{a}	33.17±2.33^{b}	*
COL (mg/dL)	173.50±9.68^{a}	141.67±2. 32^{bc}	128.67±6.03^{c}	154.67±3.77^{b}	*
HDL (mg/dL)	112.67±4.77^{a}	96.83±2.15^{bc}	87.83±3.02^{c}	101.33±3.26^{b}	*

Cont. Table 4

LDL (mg/dL)	98.67±6.07^{a}	75.67±1.33^{bc}	65.00±4.30^{c}	82.17±1.85^{b}	*
Ca (mg/dL)	109.83±3.34^{a}	104.67±1.91^{ab}	69.17±18.94^{b}	81.50±14.53^{ab}	*
P (mg/dL)	64.50±1.98	59.50±10.56	49.33±13.66	60.00±2.18	ns
TP (g/dL)	42.50±1.09^{a}	29.67±5.35^{ab}	34.67±1.38^{ab}	23.50±8.92^{b}	*
Albumin (g/dL)	90.17±24.29	98.83±17.70	96.83±19.03	128.50±5.41	ns
Glucose (mg/dL)	229.83±4.71	258.67±16.23	245.67±5.50	255.67±12.00	ns

The values are given as mean ± SEM. a, b, c: Means in the same row with different superscripts differ significantly (*: P<0.05). ns: not significant (P>0.05). ALP: Alkaline Phosphatase, TG: Triglyceride, COL: Cholesterol, HDL: High-Density Lipoprotein, LDL: Low-Density Lipoprotein, TP: Total Protein.

Bromelain supplementation had no effect on pH and aw values of the breast and drumstick meats except for day 3rd in drumstick meat (Table 5-6). Effects of dietary bromelain supplementation and storage period on microbial counts in chicken breast and drumstick meats are presented in Tables 7 and 8.

Table 5. Effects of dietary bromelain supplementations and storage period on quality parameters in the chicken breast meat.

Days	Groups	pH	a_w	TBARS *(µmolMDA/kg)*	L *	a*	b *
1	CONT	5.79±0.04	0.9897±0.001	1.29±0.06^{b}	51.70±0.61	3.55±0.47^{b}	6.88±0.62
	BRO-15	5.68±0.20	0.9919±0.000	1.25±0.18^{b}	51.23±1.67	5.40±0.49^{ab}	7.25±0.76
	BRO-30	5.78±0.21	0.9906±0.001	2.00±0.06^{a}	49.46±0.93	6.31±0.95^{a}	6.21±0.44
	BRO-45	5.71±0.06	0.9901±0.001	1.21±0.06^{b}	51.72±0.55	3.13±0.99^{b}	5.87±1.59
	p-values	ns	ns	**	ns	*	ns
3	CONT	5.72±0.05	0.9937±0.002	1.33±0.06^{b}	51.92±1.62	3.91±0.25	7.54±0.29
	BRO-15	5.67±0.00	0.9903±0.000	1.39±0.13^{b}	51.11±0.42	4.75±0.82	6.76±0.75
	BRO-30	5.86±0.31	0.9885±0.001	2.20±0.30^{a}	56.14±0.98	3.26±0.14	6.72±1.66
	BRO-45	5.63±0.17	0.9915±0.0001	1.62±0.18^{ab}	51.16±1.78	3.09±0.28	6.40±0.90
	p-values	ns	ns	*	ns	ns	ns
5	CONT	5.93±0.17	0.9894±0.002	1.73±0.08^{b}	48.21±0.49	3.59±0.73^{a}	5.17±1.31^{b}
	BRO-15	5.64±0.11	0.9877±0.001	1.87±0.24^{ab}	51.89±1.84	2.03±0.35^{b}	9.21±0.42^{a}
	BRO-30	5.69±0.00	0.9886±0.001	2.33±0.09^{a}	49.76±1.53	4.52±0.19^{a}	7.42±0.50^{ab}
	BRO-45	5.72±0.01	0.9914±0.001	1.67±0.12^{b}	49.32±0.42	3.20±0.15^{ab}	5.91±0.41^{b}
	p-values	ns	ns	*	ns	**	*
7	CONT	5.93±0.06	0.9898±0.000	2.23±0.14	48.77±0.42^{c}	2.13±0.12	6.89±0.45^{b}
	BRO-15	5.70±0.11	0.9912±0.003	2.13±0.11	49.60±1.55^{bc}	2.51±0.35	8.73±0.82^{ab}
	BRO-30	5.53±0.03	0.9905±0.001	2.38±0.04	55.23±1.82^{a}	3.09±0.38	8.97±0.95^{ab}
	BRO-45	5.40±0.13	0.9887±0.004	2.18±0.11	54.42±2.10^{ab}	2.66±0.17	10.14±0.47^{a}
	p-values	ns	ns	ns	*	ns	*
9	CONT	6.13±0.04	0.9894±0.002	2.47±0.37	49.43±1.19	3.30±0.39	5.57±1.03
	BRO-15	5.90±0.13	0.9908±0.002	2.49±0.10	52.48±1.19	2.53±0.16	8.35±1.73
	BRO-30	5.93±0.00	0.9901±0.000	2.57±0.06	52.99±0.72	3.43±0.64	10.09±0.37
	BRO-45	5.87±0.22	0.9906±0.001	2.58±0.32	53.93±1.47	2.65±0.58	6.60±0.96
	p-values	ns	ns	ns	ns	ns	ns

The values are given as mean ± SEM. a, b, c: Means in the same column with different superscripts differ significantly (*: P<0.05), (**: P<0.01).

ns: not significant (P>0.05). TBARS: Thiobarbituric acid reactive substances, L*: lightness, a*: redness, b*: yellowness. a_w: water activity.

Table 6. Effects of dietary bromelain supplementations and storage period on quality parameters in the chicken drumstick meat.

Days	Groups	pH	a_w	TBARS (*µmolMDA/kg*)	L *	a*	b *
1	CONT	6.14±0.04	0.9912±0.001	1.58±0.24	53.74±0.76^{c}	5.40±0.96	6.72±0.27^{a}
	BRO-15	5.94±0.09	0.9930±0.001	1.26±0.17	54.55±0.68^{bc}	6.61±0.09	4.72±0.54^{b}
	BRO-30	6.04±0.18	0.9918±0.002	1.75±0.19	56.75±0.80^{ab}	5.05±0.31	3.37±0.66^{b}
	BRO-45	5.83±0.06	0.9942±0.002	1.67±0.03	57.87±0.74^{a}	7.06±0.52	6.71±0.39^{a}
	p-values	ns	ns	ns	**	ns	**
3	CONT	5.93±0.16	0.9982±0.002^{a}	1.64±0.20	58.39±1.23^{a}	5.27±0.69	5.90±1.02
	BRO-15	5.95±0.16	0.9908±0.002^{b}	1.53±0.07	53.79±1.09^{b}	6.74±1.16	4.76±0.71
	BRO-30	5.77±0.05	0.9911±0.001^{b}	1.90±0.12	56.88±0.25^{b}	6.00±0.55	6.56±0.32
	BRO-45	5.75±0.14	0.9919±0.001$_{b}$	1.88±0.12	57.01±0.96^{b}	5.36±0.37	6.95±1.46
	p-values	ns	*	ns	*	ns	ns
5	CONT	6.17±0.22	0.9932±0.001	2.00±0.32	57.24±1.05^{b}	5.90±0.36^{a}	5.82±0.89
	BRO-15	5.70±0.01	0.9906±0.003	1.72±0.10	58.73±0.43^{ab}	4.18±0.20^{b}	6.34±0.36
	BRO-30	5.75±0.00	0.9889±0.000	2.39±0.17	56.56±0.60^{b}	6.99±0.46^{a}	7.39±0.95
	BRO-45	5.69±0.06	0.9881±0.000	1.88±0.15	60.56±0.69^{a}	4.15±0.72^{b}	8.69±0.29
	p-values	ns	ns	ns	**	**	ns
7	CONT	6.41±0.07	0.9917±0.000	2.38±0.04^{a}	53.37±0.29	6.21±0.38	4.90±1.03
	BRO-15	5.87±0.05	0.9923±0.000	2.28±0.11^{a}	57.73±0.56	4.64±0.37	6.41±1.62
	BRO-30	5.96±0.17	0.9920±0.000	2.45±0.16^{a}	55.73±1.54	6.04±0.67	4.95±0.77
	BRO-45	5.86±0.21	0.9925±0.000	1.85±0.11^{b}	55.05±1.03	6.14±0.49	6.44±0.47
	p-values	ns	ns	**	ns	ns	ns
9	CONT	6.38±0.08	0.9932±0.000	2.25±0.19	53.38±1.15	6.13±0.34^{ab}	3.84±0.74
	BRO-15	6.19±0.31	0.9945±0.000	2.64±0.16	56.21±0.64	4.95±0.42^{b}	5.94±2.78
	BRO-30	6.06±0.04	0.9934±0.002	2.44±0.05	57.29±2.43	7.95±0.85^{a}	8.49±1.22
	BRO-45	6.05±0.24	0.9945±0.001	2.28±0.16	55.64±1.17	5.28±0.68^{b}	6.82±0.93
	p-values	ns	ns	ns	ns	*	ns

The values are given as mean ± SEM. a, b, c: Means in the same column with different superscripts differ significantly (*: P<0.05), (**: P<0.01). ns: not significant (P>0.05). TBARS: Thiobarbituric acid reactive substances, L*: lightness, a*: redness, b*: yellowness. a_w: water activity.

Table 7. Effects of dietary bromelain supplementation and storage period on microbial counts in chicken breast meat (log cfu g^{-1}).

Days	Groups	TMAB	Coliform	*Lactobacillus* spp.	*Micrococcus-Staphylococcus*	*Pseudomonas* spp.	Yeast-Mould	TPAB
1	CONT	6.93±0.00	4.32±0.27	5.71±0.23	6.34±0.08	5.54±0.49	3.05±0.45	4.16±0.05
	BRO-15	6.14±0.28	3.77±0.43	5.11±0.09	5.64±0.51	4.83±0.53	3.24±0.16	4.36±0.60
	BRO-30	6.56±0.22	3.77±0.04	5.35±0.32	5.21±0.09	6.46±0.06	3.00±0.00	4.48±0.01
	BRO-45	6.79±0.19	3.68±0.23	5.44±0.30	4.81±0.34	4.82±0.22	3.00±0.00	4.55±0.21
	p-values	ns	ns	ns	ns	ns	ns	ns
3	CONT	7.13±0.49	4.23±0.47	6.05±0.30	6.40±0.02	6.06±0.38	3.55±0.30	5.30±0.12
	BRO-15	6.48±0.03	4.31±0.52	5.72±0.24	5.93±0.62	5.99±0.22	3.70±0.00	5.23±0.46
	BRO-30	6.42±0.12	4.06±0.06	5.56±0.15	5.45±0.12	5.07±0.59	3.59±0.64	4.61±0.53
	BRO-45	6.70±0.31	4.02±0.25	5.59±0.32	5.54±0.29	5.73±0.23	3.07±0.37	5.18±0.22
	p-values	ns	ns	ns	ns	ns	ns	ns
5	CONT	7.18±0.08	4.98±0.54	6.53±0.08	6.62±0.41	7.24±0.09^{a}	3.74±0.26	7.12±0.19^{a}
	BRO-15	5.90±0.60	4.28±0.50	5.61±0.47	5.87±0.39	5.48±0.53^{b}	4.07±0.11	5.45±0.33^{b}
	BRO-30	6.12±0.12	4.33±0.37	5.90±0.08	5.44±0.14	5.36±0.16^{b}	3.98±0.51	5.11±0.03^{b}
	BRO-45	5.87±0.27	4.27±0.15	5.70±0.18	6.30±0.30	5.53±0.03^{b}	3.48±0.00	5.50±0.12^{b}
	p-values	ns	ns	ns	ns	*	ns	**
7	CONT	7.85±0.11^{a}	5.74±0.30^{a}	6.81±0.06^{a}	6.63±0.13	3.92±0.31	7.45±0.06	7.61±0.02
	BRO-15	6.81±0.34^{b}	4.31±0.36^{b}	5.98±0.06^{b}	6.64±0.02	3.93±0.45	6.74±0.49	6.59±0.03
	BRO-30	6.57±0.03^{b}	4.87±0.12^{ab}	6.02±0.09^{b}	6.18±0.26	4.07±0.23	6.64±0.08	6.28±0.50
	BRO-45	5.69±0.21^{c}	4.27±0.05^{b}	5.39±0.09^{c}	6.49±0.02	4.09±0.03	6.64±0.22	5.60±0.00
	p-values	**	*	**	ns	ns	ns	ns
9	CONT	8.04±0.56	5.64±0.11	6.90±0.18	6.64±0.02	7.63±0.15	4.62±0.02	8.41±0.13
	BRO-15	6.30±0.30	4.86±0.14	6.50±0.38	6.62±0.34	7.58±0.20	4.94±0.10	7.90±0.10
	BRO-30	7.78±0.40	5.50±0.25	6.48±0.00	6.76±0.06	7.71±0.07	4.37±0.90	8.44±0.14
	BRO-45	8.11±0.00	5.75±0.08	5.68±0.02	6.65±0.10	7.46±0.02	4.20±0.35	7.80±0.25
	p-values	ns	ns	ns	ns	ns	ns	ns

The values are given as mean ± SEM. a, b, c: Means in the same column with different superscripts differ significantly (*: P<0.05), (**: P<0.01). ns: not significant (P>0.05). TMAB: total mesophilic aerobic bacteria, TPAB: total psychrotrophic aerobic bacteria.

Table 8. Effects of Dietary Bromelain Supplementation and Storage Period on Microbial Counts in Chicken Drumstick Meat (log cfu g^{-1}).

Days	Groups	TMAB	Coliform	*Lactobacillus* spp.	*Micrococcus-Staphylococcus*	*Pseudomonas* spp.	Yeast-Mould	TPAB
1	CONT	6.61±0.10	4.33±0.18	5.47±0.02	6.48±0.08^{a}	5.62±0.17^{a}	2.87±0.27	4.37±0.14
	BRO-15	6.55±0.42	4.27±0.11	5.84±0.29	6.28±0.04^{a}	5.97±0.09^{a}	3.15±0.15	4.88±0.23
	BRO-30	6.62±0.00	4.46±0.38	5.68±0.37	6.52±0.04^{a}	5.83±0.12^{a}	3.00±0.00	5.18±0.08
	BRO-45	6.81±0.27	3.54±0.54	5.40±0.45	5.66±0.09^{b}	4.57±0.32^{b}	3.00±0.00	4.48±0.10
	p-values	ns	ns	ns	**	*	ns	ns
3	CONT	7.18±0.03	5.18±0.07^{a}	6.34±0.11	6.68±0.20	6.16±0.08	3.16±0.16^{b}	5.28±0.04^{b}
	BRO-15	6.76±0.76	4.71±0.06^{b}	6.04±0.44	6.28±0.02	6.36±0.15	3.65±0.20^{ab}	6.06±0.03^{a}
	BRO-30	6.34±0.10	4.75±0.013^{b}	5.65±0.31	6.54±0.16	5.90±0.46	3.77±0.03^{ab}	5.47±0.17^{b}
	BRO-45	6.94±0.11	4.44±0.03^{b}	5.44±0.23	6.35±0.30	6.11±0.09	4.36±0.25^{a}	5.19±0.01^{b}
	p-values	ns	*	ns	ns	ns	*	**
5	CONT	7.44±0.15	5.53±±0.23	6.44±0.13^{a}	6.99±0.02	7.49±0.01^{a}	4.38±0.33	7.22±0.01
	BRO-15	6.77±0.29	4.68±010	6.10±0.02^{b}	6.35±0.14	6.63±0.19^{b}	3.66±0.18	6.58±0.11
	BRO-30	6.46±0.02	4.77±0.07	5.56±0.08^{c}	6.55±0.06	5.57±0.09^{c}	4.18±0.51	5.44±0.02
	BRO-45	6.41±0.18	4.48±0.27	6.16±0.02^{ab}	6.46±0.45	6.30±0.15^{b}	3.48±0.00	5.92±0.6
	p-values	ns	ns	**	ns	**	ns	ns
7	CONT	8.17±0.22	6.08±0.13	6.78±0.20	6.88±0.10	7.56±0.40^{a}	4.64±0.03^{a}	7.95±0.11
	BRO-15	6.97±0.49	4.78±0.17	6.57±0.01	6.45±0.38	7.27±0.07^{b}	3.67±0.37^{b}	7.57±0.34
	BRO-30	7.10±0.08	4.86±0.74	6.34±0.08	6.65±0.09	6.28±0.05^{d}	4.84±0.02^{a}	7.40±0.58
	BRO-45	7.35±0.03	5.24±0.47	6.15±0.11	6.75±0.16	6.99±0.07^{c}	3.56±0.01^{b}	6.92±0.13
	p-values	ns	ns	ns	ns	**	*	ns
9	CONT	8.42±0.10^{a}	6.38±0.13	7.13±0.13	7.23±0.13	7.88±0.12	4.99±0.14^{a}	8.16±0.14
	BRO-15	7.79±0.08^{ab}	5.02±0.28	6.43±0.35	6.48±0.48	7.77±0.14	4.11±0.03^{b}	8.27±0.16
	BRO-30	7.25±0.34^{b}	5.55±0.20	6.40±0.20	6.80±0.18	7.64±0.04	5.44±0.16^{a}	8.30±0.18
	BRO-45	7.16±0.12^{b}	5.47±0.29	6.25±0.26	6.74±0.41	7.59±0.33	3.85±0.15^{b}	7.60±0.20
	p-values	*	ns	ns	ns	ns	**	ns

The values are given as mean ± SEM. a, b, c: Means in the same column with different superscripts differ significantly (*: P<0.05), (**: P<0.01). ns: not significant (P>0.05). TMAB: total mesophilic aerobic bacteria, TPAB: total psychrotrophic aerobic bacteria.

Light microscopically, no significant changes were observed in the intestinal villi surface epithelium of all groups (Figure 1). Histological staining revealed a positive reaction with PAS staining of goblet cells in the villi and crypts of the duodenum, jejunum, and ileum (Figure 2). It was found that the number of these cells increased towards the ileum and this increase was parallel to the increase of the bromelain dose (Figure 2). It was determined that significantly increased (P<0.05) GCN, CD, VL, and EH which measured by histometric analysis in the duodenum, jejunum, and ileum tissues, in the bromelain groups compared to the control group (Figure 3).

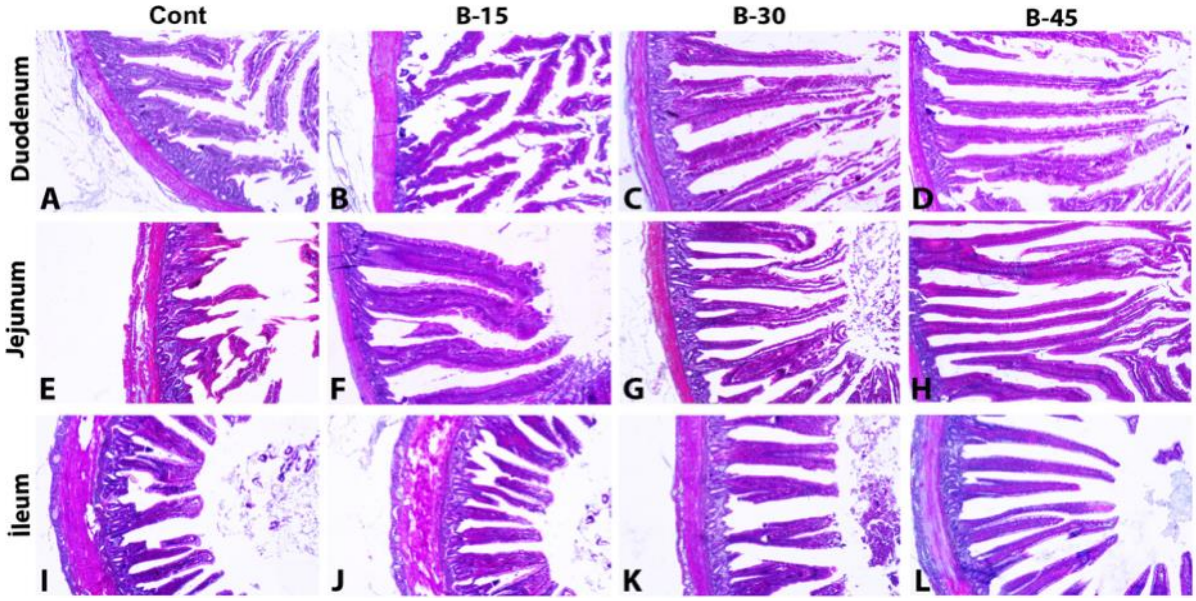

Figure 1. Image of Duodenum, Jejunum and Ileum Sections. CONT: basal diet, B-15; basal diet+15 g/kg diet of Bromelain, B-30: basal diet+30 g/kg diet of Bromelain, B-45: basal diet+45 g/kg diet of Bromelain.

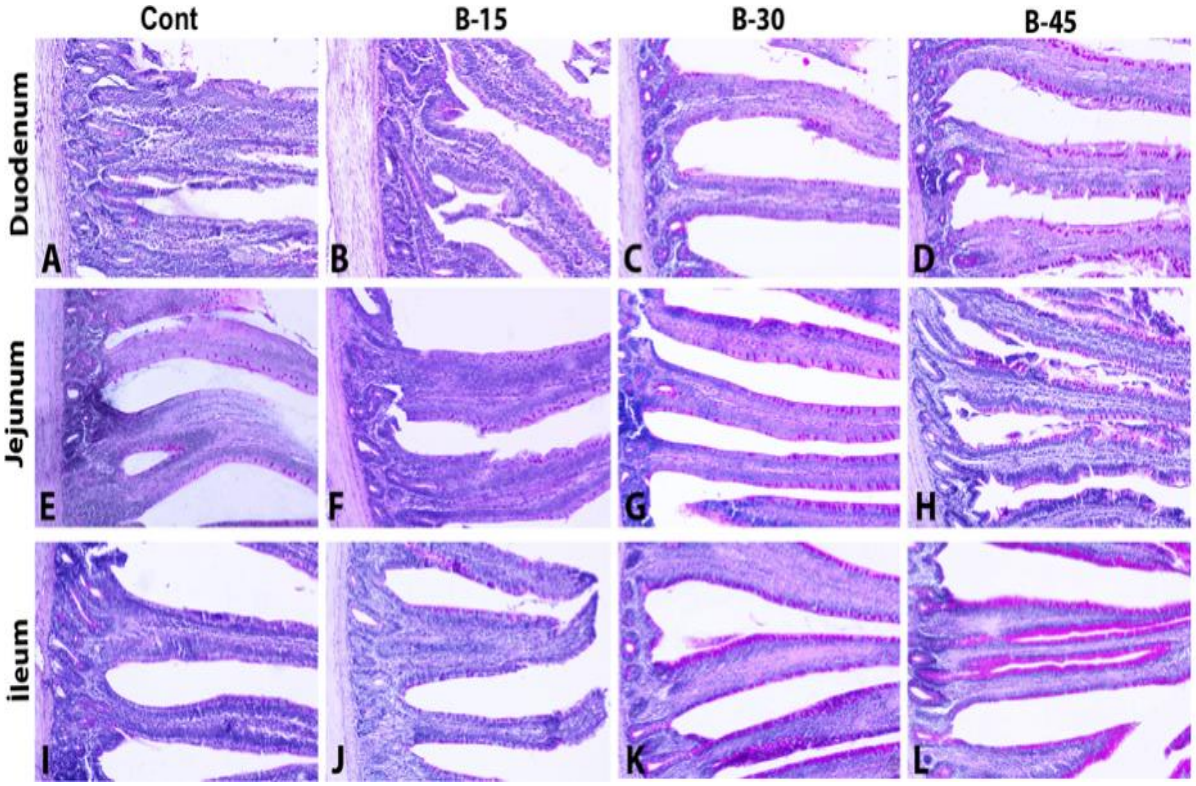

Figure 2. PAS Reaction Results of Duodenum, Jejunum, and Ileum Sections. CONT: basal diet, B-15; basal diet+15 g/kg diet of Bromelain, B-30: basal diet+30 g/kg diet of Bromelain, B-45: basal diet+45 g/kg diet of Bromelain.

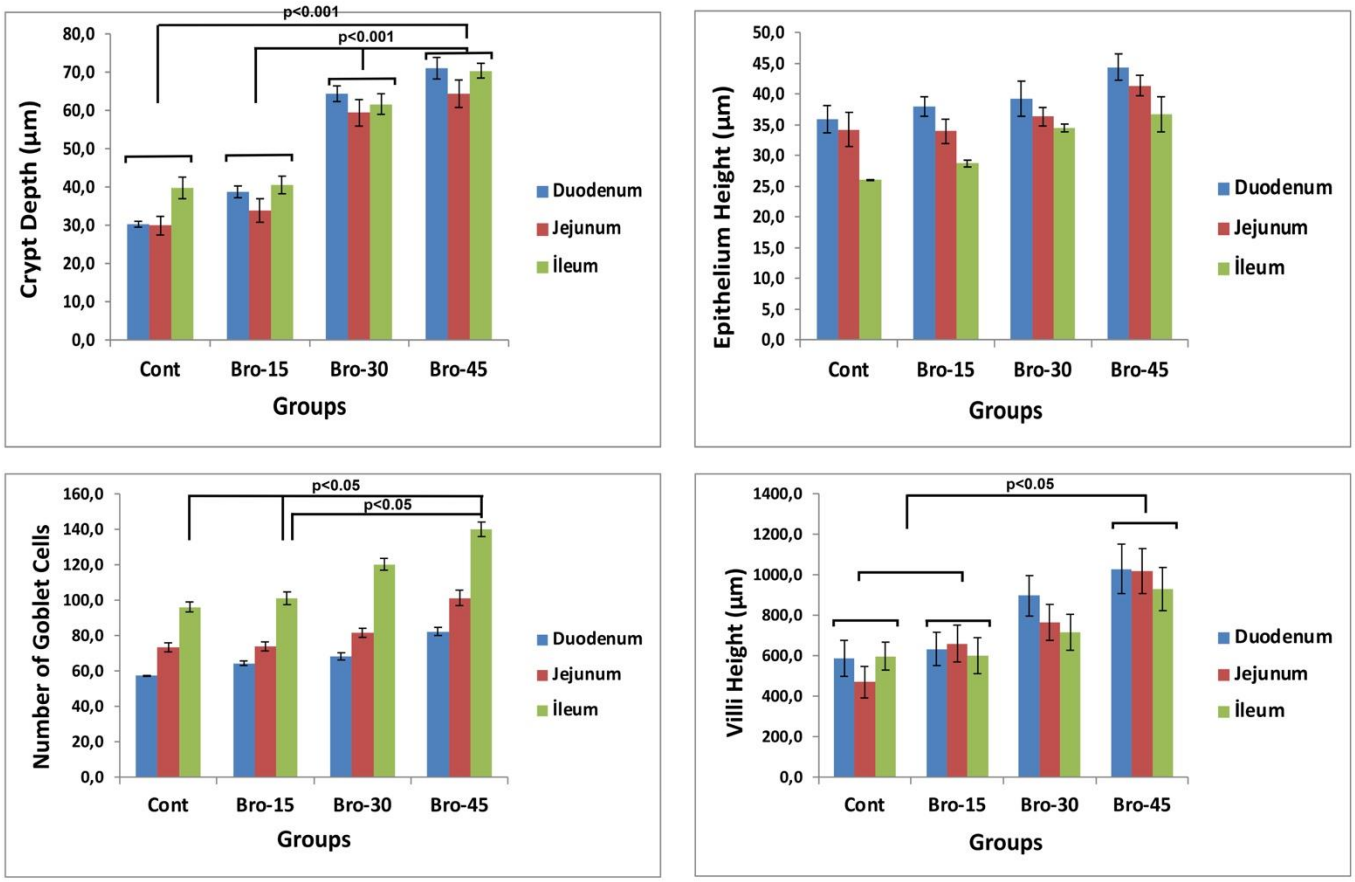

Figure 3. Histometric Measurements of Duodenum, Jejunum, and Ileum Sections. CONT: basal diet, B-15; basal 190 diets+15 g/kg diet of Bromelain, B-30: basal diet+30 g/kg diet of Bromelain, B-45: basal diet+45 g/kg diet of Bromelain. The values are given as mean ± Standard deviation. A p-value of 0.05 or lower is generally considered statistically significant.

DISCUSSION

In chickens, the development of supply organs (such as the pancreas and small intestine) accelerates shortly after hatching and the functional maturation of these organs is critical for feed utilization. Activity levels of relative amylase, total trypsin, and total and relative chymotrypsin enzymes increase with age in the pancreas [18]. Exogenous enzymes are commonly added to bird diets to increase nutrient utilization and efficiency [31]. Bromelain is a proteolytic enzyme that belongs to the cysteine peptidase family, obtained from the pineapple plant and it is considered a safe phytotherapeutic agent [2,11]. So, bromelain can be used effectively as an exogenous enzyme for improving nutrient digestibility. A previous study showed that different doses of dietary bromelain (%0.05, 0.1, 0.2, and 0.5) did not improve the BWG and FCR [16]. Also, it has been reported that pineapple (Ananas comosus) leaf powder supplements containing bromelain reduce the FCR and improve final BW [32]. In the present study, bromelain supplementation to feeds did not affect BW (except for weeks 6th), BWG (except for weeks 4th and 6th), FCR (except for weeks 3rd, 4th, and 6th), and carcass yield of broiler chicks (Table 2). However, it was observed that bromelain at a dietary dose of 30 g/kg significantly improved BW, BWG, and FCR at 6. weeks compared to the control group (Table 2).

Under normal conditions, the body produces reactive oxygen species (ROS) continuously. Antioxidant systems aid in the body's defense against ROS [33]. The antioxidant term defines as any substance that, significantly delays or inhibits the oxidation of an oxidizable substrate [34]. The SOD, GSH, and CAT are the primary enzymes that control ROS [35].

The effects of bromelain on the antioxidant-oxidant status of tissues were variable in the current investigation. The bromelain supplementation did not affect the level of GSH in all tissues and the activity of SOD in breast tissue (Table 3). Additionally, bromelain treatments increased MDA levels at different levels in breast, and drumstick tissues. These results are inconsistent with previous studies showing the antioxidant effect of bromelain. While reported that bromelain could be added to the laying hen diet up to 0.45 g/kg to boost antioxidant capacity [24], in another study Albawi [36] reported that Bromelain (20 mg\kg) as an

antioxidant could protect CYPX-induced hepatic and renal toxicity in broiler chicks. Inconsistencies in results may be due to differences in animal material and stress conditions.

Although Akit [16] reported that bromelain supplementation reduced serum ALP levels, bromelain supplementation did not affect the ALP level in the current study (Table 4). On the other hand, bromelain decreased serum HDL, LDL, and COL levels. Similarly, Yenice and coauthors [24] reported bromelain supplementation reduces serum cholesterol concentration in Layers.

Physical and chemical properties of meat affect meat quality such as color, odor, flavor, texture, and pH are the basic parameters. The biochemical processes and structural alterations that occur in the muscle within the first 24 hours after slaughter are effective in the tenderness, muscle color, quality, and flavor of the meat [37]. In post-slaughter, the pH of meat decreases due to the accumulation of lactic acid in the muscles as a consequence of anaerobic glycolysis. The pH in the muscle drops from 7.0 following slaughter to approximately 5.3–5.8 [37]. Especially pH and a_w are the main factors in meat quality. A high pH in meat is considered a sign of deterioration caused by bacterial activities [38,39]. Our study findings showed that bromelain supplementation did not have a statistically significant effect on the pH of meat. However, when compared with the control group, it is seen that the pH values of both breast and drumstick meat are numerically lower and within normal limits in the bromelain-added groups. The findings of the present study showed that bromelain has a limited and variable effect only on the color parameters and TBARS (Table 5-6). Myoglobin and hemoglobin, which are muscle pigments, affect the color of meat [40]. Compared to chicken breast meat, which has a larger percentage of white fibers, chicken leg meat has a higher concentration of red fibers [41]. As expected, the a* values of drumsticks were higher than breast meat. The amount of myoglobin denaturation and myoglobin concentration are the factors affecting the a* value. The acid environment causes the conversion of myoglobin to metmyoglobin and thus less intense color formation. [40]. Therefore, differences in a* value in breast and drumstick meats may be related to the pH value of the meat.

The microbial load is one of the factors that determine meat quality and shelf life in meat production. Certain bacteria found in meat affect its quality, diminish its shelf life and constitute a risk to human health [38]. The contamination in meat occurs primarily during processing, and as storage time is extended count of meat microorganisms increases [42]. According to research, bromelain has antibacterial properties [43] and can change gut flora [16]. Against both Gram-positive and Gram-negative bacteria, bromelain has antibacterial properties [44]. Although the exact mechanism underlying bromelain's antimicrobial effect is unknown, it is thought that bromelain may stop bacterial growth by hydrolyzing certain peptide bonds in the bacterial cell wall [43]. The current study showed that bromelain tends to reduce the microbial load of breast and drumstick meat during the storage period (Table 7-8). Results of the study showed that TMAB and Coliform loads in breast meats of bromelain-supplemented groups were significantly lower than those of the control group on the 7th day of storage. Again, on the 5th day of storage, *Pseudomonas spp.* and TPAB loads were found to be low in the bromelain-added groups. Although the Coliform load in the drumstick meats of the bromelain-added groups was not statistically significant, it was generally found to be lower than the control group.

When looking at morphological measurements It was determined that significantly increased goblet cell number, villus length, crypt depth, and epithelial height in the tissues of small intestine sections in the bromelain groups compared to the control group. The higher villus-to-crypt ratios indicate that the digestive and absorptive capacities of the villus are high [45]. A previous study showed that bromelain supplementation reduced intestinal *Escherichia coli* populations, increased Lactobacillus populations, and improved intestinal villus height in broilers [16]. Similarly, Rahman and Yang [32] reported that pineapple leaf powder which contains bromelain decreased coliform and *Escherichia coli* populations while increased the lactobacillus population. Thus, the improvements in the intestinal mucosa can be linked to the therapeutic action of the bromelain in reducing the proliferation of pathogenic bacteria and preventing possible damage to the intestinal mucosa.

CONCLUSION

In conclusion; the dietary supplementation of bromelain (30 g/kg diet) improved final BW, BWG, and FCR. The effects of bromelain treatment on the antioxidant-oxidant status of tissues were variable. Bromelain treatments increased MDA levels in breast, and drumstick tissues. On the other hand, bromelain decreased serum HDL, LDL, and COL levels. Bromelain had variable effects on meat color characteristics. However, bromelain tends to reduce the microbial load of breast and drumstick meat during the storage period. Also, bromelain improved small intestine morphology. Therefore, bromelain can be used as a supplement in the diet of broilers.

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Conflicts of Interest: The authors declare that there is no conflict of interest.

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