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Effects of Canola Bioactive Peptides on Performance, Digestive Enzyme Activities, Nutrient Digestibility, Intestinal Morphology and Gut Microflora in Broiler Chickens

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Abstract

The aim of the present study was to evaluate the effects of canola bioactive peptides (CBP) produced by enzymatic hydrolysis of canola meal on performance, digestive enzyme activity, nutrient digestibility, gut microflora and intestinal morphology of broiler chickens. A total of 250 one-day-old Ross 308 male broiler chicks were randomly allocated to 5 dietary treatments with 5 replicates of 10 birds per each. Birds were fed with a basal diet (Control) or a basal diet supplemented with CBP at 100, 150, 200 and 250 mg/kg over 42 days. Results indicated that addition of 200 and 250 mg CBP/kg diet increased ($P < 0.05$) body weight gain and decreased feed conversion ratio (1-28 d and 29-42 d) ($P < 0.05$). Adding 200 and 250 mg CBP/kg diet improved amylase, lipase and protease activities in the small intestine ($P < 0.05$). Also, adding CBP to diet increased dry matter, organic matter, crude protein and ether extract digestibility ($P < 0.05$). The villus height, the ratio of villus height to crypt depth of duodenum, jejunum, and ileum in chicks fed by different amounts of CBP increased while crypt depth significantly decreased ($P < 0.05$). Adding CBP decreased gram negative bacteria counts in ileum and caecum compared to the control group. Results of the present study suggest that adding CBP to broiler diet may increase nutrient digestibility and act as an antimicrobial agent.

Introduction

Bioactive peptides are food protein hydrolysates produced by microbial fermentation, enzymatic digestion (or in vitro enzymatic proteolysis), and alkali or acid hydrolysis. They perform physiological functions by induction of beneficial biological process (Korhonen and Pihlanto, 2006; Hartmann and Meisel, 2007; He *et al.*, 2013) such as antioxidative, antimicrobial, antihypertensive, cytomodulatory and immunomodulatory activities (Hartmann and Meisel, 2007; Yang *et al.*, 2009). Bioactive peptides can also act as antimicrobial agents by interacting with microbial host invaders or by suppressing or stimulating certain immune responses (Hancock and Sahl, 2006).

Enzymatic hydrolysis is widely used in food industry for the elimination of allergens, production of flavors, detoxification, and improvement of nutrient quality (Lahl and Braun, 1994; Rolle, 1998; Dust *et al.*, 2005; Sathe *et al.*, 2005). This process affects protein structure by cleaving to oligopeptides, smaller peptides,

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and free amino acids (Kamnerdpetch *et al*., 2007). During the hydrolysis process, peptides of different length and different amino acid residue compositions are produced and can exhibit a variety of functional properties (Alashi *et al*., 2013). The produced peptides generally contain 2–20 amino acid residues depending on their size, amino acid composition, and sequence, leading to different bioactivities and functionalities (Dziuba *et al*., 1999; Pihlanto-Leppälä, 2001).

Currently, low molecular weight peptides and free amino acids are used as high-protein feed ingredients and palatability-enhancing agents in animal diets (Dust *et al*., 2005; Folador *et al*., 2006; Nechienzia *et al*., 2010). Several studies have been shown that canola peptides possess different bioactivities (Pan *et al*., 2011; Aachary and Thiyam, 2012) and health/nutritional functions and therefore, might be useful as functional ingredients to improve broilers growth performance. Little information is available on the use of canola bioactive peptides (CBP) as a functional ingredient in broiler diet. The purpose of the present study was to evaluate the effects of different levels CBP produced by enzymatic hydrolysis on performance, digestive enzyme activities, nutrient digestibility, intestinal morphology and gut microflora in broiler chickens.

Material and Methods

Preparation of canola protein isolate

The canola protein isolate was produced from defatted canola meal following method described by Alashi *et al.* (2014) with some modifications. Defatted canola meal was suspended in distilled water (1:10 w/v), adjusted to pH=12.0 with 0.1 M NaOH and extracted by stirring for 1 hrs using an overhead stirrer equipped with a propeller (IKA® RW 20 D CHN, Staufen, Germany) and then centrifuged at 18°C and 3000 × g for 10 min. The residue of centrifugation process was extracted twice with the same volume of 0.1 M NaOH. The supernatants were pooled, adjusted to the isoelectric point (pH=4.0) using 0.1 M HCl solution, centrifuged (3000 × *g* for 10 min), and the precipitate recovered. The precipitate was washed with distilled water, adjusted to pH=7.0 using 0.1 M NaOH and then lyophilized (Labconco Corp., Kansas City, MO, USA). The protein isolate powder was placed in a polyethylene bag and stored at -20°C until further analysis.

Production of Canola Bioactive Peptides

The CBP were produced according to method described by Alashi *et al.* (2014) with some modifications. Canola protein isolate was dispersed and distilled in a reactor placed on a C-MAG HS7 magnetic stirrer (IKA-Werke GmbH & Co. KG, Staufen, Germany) and then hydrolyzed using Alcalase (pH=8.0 and 60°C) at an enzyme-substrate ratio of 1:20 for 4 hrs to obtain protein hydrolysate solution. pH was adjusted with 1 M NaOH in a temperature-controlled water bath. After 4 hrs, the enzyme was inactivated by heating to 90°C for 15 min. The resulting hydrolysate solution was centrifuged at 8000 × *g* for 10 min at 4°C and then lyophilized. The powder was placed in polyethylene bags and stored at -20°C until further analysis.

Determination of concentration and molecular weight distribution of CBP

The molecular weight distribution of CBP was determined using TSK gel 3000 PWXL columns (Tosoh, Japan) coupled with an HPLC system (Agilent 1100, Agilent Technologies Inc., Santa Clara, CA, USA). The acetonitrile in water (1:1, v/v) containing TFA (0.1%, v/v) was used as the mobile phase. The absorbance was monitored at 225 nm with a flow rate of 0.5 mL/min. Bovine serum albumin (BSA, MW: 66,000 Da), cytochrome C (MW: 12,384 Da), bacitracin (MW: 1423 Da), and reduced glutathione (GSH, MW: 307 Da) were used as the molecular weight standards. The protein concentration of lyophilized CBP was measured by the Biuret method (Gornall *et al*., 1949) using BSA as a standard.

Experimental design and animal husbandry

All of procedures, animal ethics and welfare were carried out in accordance with guidelines set out by Sari University of Agricultural Sciences and Natural Resources, Sari, Iran.

A total of 250 one-day-old Ross 308 male broiler chicks were randomly distributed to 25 experimental pens. There were five treatments with five replicates of 10 chicks. All birds were reared for 42 d according to the recommended standard guidelines of Ross 308 (Ross 308, 2014). The nutrient requirements of broilers were met according to Ross 308 recommendations (Ross 308, 2014; Table 1).

Table 1. Ingredients and composition of the basal diet (g/kg)

Ingredients	Starter (1–10 d)	Grower (11-28 d)	Finisher (29–42 d)
Corn	522.4	562.8	663.5
Soybean meal	410.1	361.5	259.9
Soybean oil	23.5	37.7	35.7
Sodium bicarbonate	0.00	1.0	1.0
Oyster shell	12.3	11.2	11.6
Dicalcium phosphate	18.2	15.8	17.2
DL-Methionine	2.8	1.8	1.7
L-Lysine-HCl	2.1	0.40	1.5
Vitamin premix[1]	2.5	2.5	2.5
Mineral premix[2]	2.5	2.5	2.5
Salt	3.6	2.8	2.9
Chemical composition			
ME_n (Kcal/kg)	2880	3000	3100
Crude protein	225.00	210.00	175.10
Methionine	6.64	5.45	4.82
Methionine + Cystine	10.50	9.00	7.80
Lysine	13.90	11.40	9.70
Available phosphorus	4.80	4.40	4.30
Calcium	9.64	8.80	8.60
Sodium	1.60	1.54	1.54

[1]Vitamin premix supplied the following per kilogram of diet: vitamin A, 9,000 IU; vitamin D_3, 2,000 IU; vitamin E, 18 IU; vitamin K_3, 2 mg; B_1, 2 mg; B_2, 6.6 mg; B_6, 3 mg; vitamin B_{12}, 0.015 mg; niacin, 30 mg; choline chloride, 250 mg; calcium D-pantothenate, 10 mg; folic acid, 1mg.
[2]Mineral premix supplied the following per kilogram of diet: Mn, 100 mg; Fe, 50 mg; Zn, 85 mg; Cu, 10 mg; I, 1 mg, Se, 0.2 mg.

The experimental diets were isocaloric and isonitrogenous and consisted of adding 0, 100, 150, 200 or 250 mg of CBP/kg of diet to the basal diet. Molecular weight distribution of peptides was determined using gel filtration chromatography (Table 2). Feed and water were supplied *ad libitum* throughout the experiment.

Digestive enzyme activities

Amylase activity was determined using previously established methods (Somogyi, 1960). One unit of amylase activity was defined as the amount of amylase that caused the formation of reducing power equivalent to 1 mg of glucose in 30 min per mg of intestinal digesta protein at 38°C. Corn starch was used as the substrate. Lipase activity was assayed (Tietz and Fiereck, 1966) using olive oil as a substrate. Finally, protease activity was determined using method of Lynn and Clevette-Radford (1984) in which Azocase was the substrate.

Determining nutrient digestibility

To determine nutrient digestibility, 3 g/kg chromic oxide (Cr_2O_3) was added to experimental diets as a marker (Sales and Janssens, 2003). On day 35, two chicks per pen were selected and placed in battery cages with wire mesh bottom and excreta collection trays. Feed and excreta samples were subsequently analyzed for dry matter (DM), organic matter (OM), ether extract (EE), and crude protein (CP) (AOAC, 2006).

Intestinal morphology

On day 42, two broilers per pen were killed, and intestinal segments were removed as follows: 1) the duodenum was the segment from the gizzard to pancreatic and bile ducts, of which the middle section was taken for microscopy; 2) the jejunum spanned the point of entry of the bile ducts and Meckel's diverticulum; and 3) the ileum was 10 cm proximal to the ileo-cecal junction. The samples were flushed with physiological saline and fixed in 10% formalin. Samples were stained with hematoxylin and eosin and villus height and crypt depth were measured according to Xia *et al*. (2004). The slices were observed with an electron microscope (CK 40, Olympus, Tokyo, Japan). Morphological indices were measured using image processing and analysis system (Version 1, Leica Imaging System Ltd, Cambridge, UK).

Intestinal microflora

The bacteriological examination was conducted at the end of the experiment according to

methods used by Wen and He (2012). One chick per pen with body weight as close as possible to mean pen weight was chosen and slaughtered. Ileal and caecal digesta were then collected to measure total bacteria count and gram-negative bacteria according to procedures used by Macfaddin and Jean (2000). Further examinations were done according to Wen and He (2012).

Statistical analysis

Using a completely randomized design with five treatments and five replicates per treatment, the data were analyzed using the General Linear Model (GLM) of SAS Institute (SAS, 2004): $Y_{ij}= \mu + T_i + e_{ij,}$ where Y_{ij} is the value of each observation, μ is observations mean, T_i is the effect of treatment and e_{ij} is the effect of random errors. Means of treatments were compared by Duncan's multiple range tests (1955). Differences were considered significant at $P < 0.05$. The effects of dietary CBP levels on the dependent variables were performed using regression analysis.

Results

Molecular weight distribution of CBP

Production of CBP by enzymatic digestion yielded molecular weights (MW) of CBP in the range of 180-3000 Da. The main fraction of peptides (54.9%) was constituted by di-/tripeptides (MW 180-500 Da), followed by oligopeptides/polypeptides (39.3%; MW 500 to >3000 Da), and free amino acids (5.8%, MW< 180 Da) (Table 2).

Table 2. Molecular weight distribution of CBP

Molecular weight range (Da)	Peptide fraction (%)
>3000	0.09 ± 0.04
3000-2000	0.89 ± 0.33
2000-1000	11.63 ± 0.36
1000-500	26.69 ± 0.19
500-180	54.86 ± 0.49
<180	5.81 ± 0.76

Data are presented from mean values of the duplicate sample as Mean ± Standard deviation.

Growth performance

Addition of CBP at 200 and 250 mg/kg diet improved ($P < 0.05$) body weight gain (BWG) and decreased ($P < 0.05$) feed conversion ratio (FCR) in the period of 1-28 and 29-42 d relative to the other groups (Table 3). Among all dietary treatments, broiler chicks that received 250 mg CBP/kg diet had the highest BWG and the lowest FCR ($P < 0.05$). Dietary CBP addition had a linear and quadratic effect ($P < 0.0001$) on BWG and FCR. There were no significant differences among experimental treatments for FI.

Table 3. Effects of different levels of CBP on growth performance in broiler chicks

	Dietary treatments[1]					*SEM*[2]	*P*-value		
	CBP 0	CBP 100	CBP 150	CBP 200	CBP 250		Anova	Linear	Quadratic
Body weight gain (g)									
1-28 d	1110[c]	1121[c]	1122[c]	1190[b]	1230[a]	9.931	<0.05	<0.0001	<0.0001
29-42 d	1076[c]	1076[c]	1103[c]	1141[b]	1189[a]	10.169	<0.05	<0.0001	0.0350
Feed intake (g)									
1-28 d	1616	1634	1606	1609	1629	4.202	0.1363	<0.0018	0.6528
29-42 d	2244	2239	2278	2265	2276	13.780	0.8762	0.001	0.7158
Feed conversion ratio									
1-28 d	1.46[a]	1.46[a]	1.43[a]	1.35[b]	1.32[c]	0.012	<0.05	<0.0001	0.0195
29-42 d	2.09[a]	2.08[a]	2.06[ab]	1.99[bc]	1.92[c]	0.017	<0.05	0.0002	0.0516

[a-d]Means with different superscripts in the same row differ significantly ($P < 0.05$).

[1]CBP 0: basal diet; CBP 100: basal diet plus 100 mg of canola peptides per kg; CBP 150: basal diet plus 150 mg of canola peptides per kg; CBP 200: basal diet plus 200 mg of canola peptides per kg and CBP 250: basal diet plus 250 mg of canola peptides per kg.

[2] standard error of the means.

Digestive enzyme activities

The addition of 200 and 250 mg CBP/kg diets improved intestinal amylase, lipase and protease activities (Table 4). CBP supplementation increased intestinal amylase, lipase, and protease activities in a dose-dependent manner (Table 4).

Nutrient digestibility

Among all dietary treatments, broiler chicks that received 200 and 250 mg CBP/kg organic matter (OM) and ether extract digestibilities increased ($P < 0.0001$; Table 5). Compared to the control group, dry matter (DM) and crude protein digestibilities were significantly improved in birds fed CBP diet ($P < 0.0001$; Table 5). CBP improved DM, OM, CP, and EE digestibilities in a positively linear manner ($P < 0.0001$).

Table 4. Effects of different levels of CBP on intestinal enzyme activity in broiler chickens at 42 d (U/mg of digesta protein)

	Dietary treatments[1]					*SEM*[2]	*P*-value		
	CBP 0	CBP 100	CBP 150	CBP 200	CBP 250		Anova	Linear	Quadratic
Amylase[3]	8.45[c]	8.52[c]	8.80[bc]	9.74[b]	10.54[a]	0.226	0.0031	0.0002	0.1491
Lipase[4]	19.50[d]	19.27[d]	20.54[c]	21.82[b]	23.67[a]	0.359	<0.0001	<0.0001	0.0030
Protease[5]	78.75[c]	80.90[bc]	79.02[c]	87.52[b]	90.64[a]	1.354	0.0034	0.0004	0.1638

[a-d]Means with different superscripts in the same row differ significantly ($P < 0.05$).
[1] CBP 0: basal diet; CBP 100: basal diet plus 100 mg of canola peptides per kg; CBP 150: basal diet plus 150 mg of canola peptides per kg; CBP 200: basal diet plus 200 mg of canola peptides per kg and CBP 250: basal diet plus 250 mg of canola peptides per kg.
[2] standard error of the means.
[3] Amylase activity unit (1 Somogyi unit) was defined as the amount of amylase that would cause the formation of reducing power equivalent to 1 mg of glucose in 30 min at 40°C/mg of intestinal digesta protein.
[4] Lipase activity unit (Sigma-Tietz units) was equal to the volume (mL) of 0.05 *M* NaOH required neutralizing the fatty acid liberated during 6 hrs incubation with 3 mL of lipase substrate at 37°C/mg of intestinal digesta protein.
[5] Protease activity unit was defined as mg of azocasein degraded during 2 hrs incubation at 38°C/mg of intestinal digesta protein.

Intestinal morphology

CBP altered intestinal morphology of chickens (Table 6). Intestinal villus height and the ratio of villus height: crypt depth of the duodenum, jejunum, and ileum in chickens fed 250 mg CBP/kg were higher than other groups. The addition of 200 and 250 mg CBP/kg decreased crypt depth compared to the other treatments. Dietary CBP addition had a linear effect ($P < 0.0001$) on the villus height, crypt depth, and villus height: crypt depth in the duodenum, jejunum and ileum.

Intestinal microflora

CBP diets reduced total bacteria and gram-negative bacteria counts in the ileum and caecum ($P < 0.0001$; Table 7). The lowest total bacteria and gram-negative bacteria counts in ileum and caecum were observed in the chickens with the diets of 250 mg CBP/kg of diet. Results showed a linear increase ($P < 0.0001$) in total bacteria and gram-negative bacteria counts in the ileum and caecum of birds fed CBP diets.

Discussion

Results indicated that addition of 200 and 250 mg CBP/kg diet leads to linear and quadratic increase in BWG and similar decreases in FCR. Wang (2005), Wang *et al.* (2011) and Mateos *et al.* (2014) reported that BWG and FCR improved in broiler chickens fed soybean peptides. Results revealed that inclusion of CBP leads to linear increases in intestinal amylase, lipase and protease activities. The effects were most noticeable in birds fed 200 and 250 mg CBP/kg diet. Hong *et al.* (2004) showed that fermentation increased protein content, eliminated trypsin inhibitors, and reduced peptide size in soybean seed and soybean meals. These may make soybean meals a useful ingredient in livestock diets. Large peptides, such as antigenic proteins, could be hydrolyzed to become small peptides during fermentation. Therefore, the improvement in intestinal enzyme activities in broilers fed fermented soybean meal may be associated with degradation of soybean globulin (Feng *et al.*, 2007).

Table 5. Effects of different levels of CBP on nutrient digestibility in broiler chickens at 42 d (%)

	Dietary treatments[1]					*SEM*[2]	*P*-value		
	CBP 0	CBP 100	CBP 150	CBP 200	CBP 250		Anova	Linear	Quadratic
DM	70.64[b]	74.5[a]	74.64[a]	74.86[a]	74.6[a]	0.378	<0.0001	<0.0001	0.0001
Organic matter	71.5[b]	71.7[b]	71.9[b]	74.0[a]	74.3[a]	0.271	<0.0001	<0.0001	0.0419
CP	62.74[b]	67.8[a]	67.6[a]	67.7[a]	67.9[a]	0.435	<0.0001	<0.0001	<0.0001
Ether extract	70.32[b]	65.66[c]	61.5[d]	71.06[b]	76.54[a]	1.078	<0.0001	<0.0001	<0.0001

[a-d]Means with different superscripts in the same row differ significantly ($P < 0.05$).
[1] CBP 0: basal diet; CBP 100: basal diet plus 100 mg of canola peptides per kg; CBP 150: basal diet plus 150 mg of canola peptides per kg; CBP 200: basal diet plus 200 mg of canola peptides per kg and CBP 250: basal diet plus 250 mg of canola peptides per kg.
[2] standard error of the means.

Table 6. Effects of different levels of CBP on intestinal morphology in broiler chickens at 42 d

	Dietary treatments[1]					*SEM*[2]	*P*-value		
	CBP 0	CBP 100	CBP 150	CBP 200	CBP 250		Anova	Linear	Quadratic
Villus height (µm)									
Duodenum	1857.61^{b}	1861.50^{b}	1867.50^{b}	1995.75^{a}	2007.50^{a}	17.928	0.0006	<0.0001	0.1950
Jejunum	1740.43^{c}	1748.35^{c}	1833.25^{b}	1838.50^{b}	1927.50^{a}	15.837	<0.0001	<0.0001	0.2385
Ileum	1582.25^{b}	1630.83^{b}	1583.00^{b}	1595.25^{b}	1775.75^{a}	16.706	0.0001	<0.0001	0.0001
Crypt depth (µm)									
Duodenum	199.36^{a}	191.60^{ab}	185.50^{bc}	178.50^{c}	178.25^{c}	2.231	0.0025	0.0001	0.3198
Jejunum	193.50^{a}	187.25^{b}	181.50^{c}	173.00^{d}	170.75^{d}	1.899	<0.0001	<0.0001	0.4578
Ileum	172.00^{ab}	175.50^{a}	171.25^{ab}	165.50^{c}	168.75^{bc}	0.972	0.0067	0.0059	0.7558
Villus height: Crypt depth									
Duodenum	9.35^{c}	9.72^{bc}	10.07^{b}	11.18^{a}	11.29^{a}	0.184	<0.0001	<0.0001	0.8090
Jejunum	8.99^{d}	9.34^{d}	10.10^{c}	10.63^{b}	11.29^{a}	0.182	<0.0001	<0.0001	0.4910
Ileum	9.21^{b}	9.29^{b}	9.25^{b}	9.64^{b}	10.52^{a}	0.119	<0.0001	<0.0001	0.0020

[a-d]Means with different superscripts in the same row differ significantly ($P < 0.05$).

[1] CBP 0: basal diet; CBP 100: basal diet plus 100 mg of canola peptides per kg; CBP 150: basal diet plus 150 mg of canola peptides per kg; CBP 200: basal diet plus 200 mg of canola peptides per kg and CBP 250: basal diet plus 250 mg of canola peptides per kg.

[2] standard error of the means.

Feng *et al.* (2007) found that fermented soybean meal significantly increased the activation of intestinal trypsin, lipase and protease enzymes in broilers during starter period ($P < 0.05$) and enhanced the protease activity in broilers during grower ($P < 0.05$), while amylase activity was not affected in both feeding periods. In our experiment, adding CBP at 200 and 250 mg/kg diet increased DM, OM, CP, and EE digestibility in a linear manner ($P < 0.0001$). Kiers *et al.* (2003) showed an increase in nutrient solubility and digestibility in vitro from in fermentation of soybean (Kiers *et al.*, 2003). Contrary to the present findings, Jin *et al.* (2009) reported no effects of dietary supplementation of the potato antimicrobial peptide (AMP) on DM and CP digestibility in weanling pigs. Increase nutrient retention in broilers fed on diets supplemented with AMP may be due to the modulation of gut environment, improvement of beneficial intestinal microbial balance, improved small intestinal morphology or stimulation of the mucosal immune system (Jin *et al.*, 2008; Tang *et al.*, 2009; Ohh *et al.*, 2010).

Table 7. Effects of different levels of CBP on the ileal and caecal bacterial count (log10 CFU) in broiler chickens at 42 d

	Dietary treatments[1]					*SEM*[2]	*P*-value		
	CBP 0	CBP 100	CBP 150	CBP 200	CBP 250		Anova	Linear	Quadratic
Ileum: Total bacteria	8.19^{a}	7.94^{a}	7.30^{b}	7.02^{c}	6.15^{d}	0.152	<0.0001	<0.0001	0.0220
Gram negative bacteria	7.58^{a}	6.87^{b}	6.67^{b}	6.58^{b}	5.62^{c}	0.136	<0.0001	<0.0001	0.2247
Caecum: Total bacteria	8.49^{a}	8.12^{b}	7.83^{c}	7.64^{c}	6.54^{d}	0.139	<0.0001	<0.0001	0.1414
Gram negative bacteria	7.94^{a}	7.27^{b}	7.21^{b}	7.11^{b}	6.05^{c}	0.126	<0.0001	<0.0001	0.0005

[a-d]Means with different superscripts in the same row differ significantly ($P < 0.05$).

[1] CBP 0: basal diet; CBP 100: basal diet plus 100 mg of canola peptides per kg; CBP 150: basal diet plus 150 mg of canola peptides per kg; CBP 200: basal diet plus 200 mg of canola peptides per kg and CBP 250: basal diet plus 250 mg of canola peptides per kg.

[2] standard error of the means.

Villus height and crypt depth of duodenum, jejunum and ileum are indicative of gut health. Therefore, it was important to analyze the intestinal morphology to elucidate possible mechanisms of growth promotion. In this experiment, supplementation of broiler diets with 200 and 250 mg CBP/kg increased villus height, villus height: crypt depth, and decreased crypt depth of duodenum, jejunum, and ileum in a linear manner ($P < 0.0001$). In line with the present study, Bao *et al.* (2009) reported an increase in villus height in the duodenum and

jejunum in broiler chickens given diets supplemented with AMP. In another study, an increase of villus height in the duodenum and jejunum, as well as an increase in villus height: crypt depth, were observed in broilers fed diet supplemented with 10% fermented grapeseed meal (Xu *et al.*, 2012). Liu *et al.* (2008) reported that birds given a diet supplemented with AMP had greater villus height in the duodenum and jejunum but not in the ileum. In another study, birds fed 90 mg/kg AMP-A3 diet had greater villus height in the duodenum, jejunum, and ileum than birds fed the control diet, while dietary treatments had no significant effects on crypt depth of the duodenum, jejunum and ileum (Choi *et al.*, 2013). Similarly, supplementing basal diets with AMP had a positive linear effect on villus height and villus height: crypt depth ratio, but a negative linear effect on crypt depth of the duodenum and ileum (Wen and He, 2012). Small peptides increase the number and size of villus in the small intestine when compared with other intact proteins. An increase in the number and size of villus increases the amount of surface area available for nutrient absorption, which in turn can improve the efficiency of growth performance (McCalla *et al.*, 2010).

The present study also indicated that adding CBP to diet linearly affected ($P < 0.0001$) intestinal gram-negative bacterial count. The lowest total and gram-negative bacteria count in ileum and caecum were found in birds fed on 250 mg CBP/kg. Gram-negative bacteria were found to be evident in the ileum and caecum in the control group, indicating that CBP had an anti-bacterial effect. This is in agreement with an experiment conducted by Wen and He (2012). Intestinal harmful bacteria may induce intestine inflammation by releasing metabolites, such as lipopolysaccharide from gram-negative bacteria and lipoteichoic acid (a major constituent of the cell wall of gram-positive bacteria) (Sukhotnik *et al.*, 2002; Niewold, 2007). The metabolites may bind to a protein and subsequently to CD14 to activate inflammatory responses through initiating toll-like receptors 4 and 2 on macrophages (Scott *et al.*, 2000 and Niewold, 2007). The harmful microbiota hydrolyses bile salts which are required for proper fat digestion and absorption, and competes with the host for the uptake of nutrients and energy, thus decreasing fat, protein and energy efficiency (Dibner and Richards, 2005). By decreasing gram-negative bacterial counts in the ileum and caecum, CBP increased nutrients digestion and absorption and intestinal enzyme activity. However, the effect of CBP on decreasing gram-negative bacteria needs to be further investigated.

Conclusion

Results of the present study showed that CBP obtained from enzymatic hydrolysis by Alcalase enzyme mainly consisted of di-and tri-peptides and oligo peptides. The growth performance, digestive enzyme activities, nutrients digestibility, intestinal morphology and intestinal microflora were significantly improved in chickens fed with diets containing 200 and 250 mg/kg CBP. Adding CBP to broiler diets has the potency to decrease harmful intestinal microflora and can be used as an antimicrobial additive.

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