Protease supplementation attenuates the intestinal health damage caused by low-protein diets in Pekin ducks

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ABSTRACT The objective of this study was to investigate the effects of low-protein diets with low digestibility of feed ingredients on intestinal damage and to explore whether the protease supplementation can alleviate the damage in Pekin ducks. A total of 576 Pekin ducklings (6 replicate pens, 16 ducks/pen) were randomly assigned to 6 dietary treatments (3 × 2 factorial arrangement) in a randomized complete block design. Factors were CP levels (13.5%, 15.5%, and 17.5%) and protease (0 or 20,000U/kg). Compared with the diets containing 17.5% CP, low-protein diets (13.5% CP) showed suppressed ($P < 0.05$) growth performance and feed intake (FI); reduced ($P < 0.05$) serum-free arginine, isoleucine, leucine, methionine, phenylalanine, valine, and proline as well as the cecal acetate and propionate concentration; increased ($P < 0.05$) plasma and ileal mucosal tumor necrosis factor-α (**TNF-α**) concentration; and downregulated ($P < 0.05$) mRNA expression of *TNF-α, nuclear transcription factor-κb, interferon gamma,* and *Occludin* in ileal mucosa. Irrespective of the dietary CP levels, protease supplementation significantly increased ($P < 0.05$) the serum-free glutamic acid concentration while decreasing ($P < 0.05$) the plasma endotoxin, IL-6, and the cecal isovalerate concentration. A significant interactive effect was observed between low-protein diets and protease supplementation ($P < 0.05$) on serum-free arginine concentration, the ratio of ileal villus height to crypt depth, and the IL-6 concentration in ileal mucosa. These results indicated that low-protein diets could damage intestinal integrity to induce systemic inflammation response and at last to suppress growth performance. Protease supplementation could partly attenuate the negative effects on gut health caused by low-protein diets in Pekin ducks.

Key words: low-protein diet, intestinal health, Pekin duck, protease, serum free amino acid

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INTRODUCTION

Reduced-CP diets have potential benefits, such as reduction of environmental impact and the feeding cost, but the challenge lies in maintaining the digestive function and growth performance of the birds. A low-protein diet refers to a diet that has a 2% to 4% reduction in CP levels from the NRC (2012)-recommended levels required to maintain animal health without adverse nutritional effects and also to improve the nitrogen (**N**) deposition in livestock (Tilg et al., 2015). Therefore, using low-protein diets in the poultry industry needs more concern for intestinal and body health. Barekatain et al. (2019) found that feeding birds with low-protein diets (17% grower phase/15% finisher phase) compared with the diets with higher protein (22% grower phase/21% finisher phase) may lead to a higher intestinal permeability. Chen et al. (2016) also showed that reducing dietary protein from 26% to 18%, without supplementing all the essential amino acids (**EAA**) exacerbated the effect of aflatoxicosis on broiler's performance and nutrient utilization, with an increase in intestinal permeability. A higher intestinal permeability and inflammatory response increased the nutrient requirements of broilers (Humphrey and Klasing, 2004), indicating that low-protein diets aggravate the damage of intestinal health.

On the contrary, Macelline et al. (2019) found that broilers raised under poor sanitary conditions and fed with a low-protein diet (18% CP at 1–14 d/17% CP at

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15–35 d) supplemented with synthetic amino acids (**AA**) (L-lysine, L-methionine [**Met**], L-threonine, L-tryptophan, L-valine [**Val**], L-arginine [**Arg**], L-isoleucine [**Ile**], L-glycine, L-serine) maintained the growth performance, intestinal integrity, and reduced N excretion. Meanwhile, many studies manifested that surplus dietary proteins can be fermented by the resident microbiota in the ileum and cecum to yield a greater diversity of end products, including short-chain fatty acids (**SCFA**), branch-chain fatty acids (**BCFAs**), amines, phenols, indoles, thiols, CO_2, H_2, and H_2S, many of which have toxic properties (Qaisrani et a., 2015). Wilkie et al. (2005) showed that 10% to 43% of the undigested proteins consumed by the broilers are subjected to fermentation by cecal bacteria. These studies indicate that reducing dietary CP levels and undigested protein contents in the ileum or cecum may benefit the intestinal health of birds. However, to our best knowledge, there is little information on the effect of low-protein diets on intestinal health and cecal fermentation products in Pekin ducks.

Protease supplementation may improve the utilization of dietary proteins and AA (Olukosi et al., 2007; Freitas et al., 2011). Therefore, the nutritionists can use protease supplement to formulate lower levels of protein diets to maintain the growth performance, while promoting the sustainability of poultry production (Leinonen and Williams, 2015). Cowieson et al. (2018) further found that exogenous protease with ascorbic acid had beneficial effects on broiler feed conversion, AA digestibility, and intestinal integrity. Previous researchers have demonstrated that exogenous proteases may contribute to the shifting of substrates available in the intestine for bacterial growth (Malo et al., 2010). The mechanism by which protease contributes to positive gut health is not entirely clear. Therefore, our study aimed to investigate the effect of low-protein diets and protease supplementation on intestinal health in Pekin ducks by analyzing serum AA profile and inflammatory factors content, intestinal development and morphology, intestinal inflammation and barrier function along with the cecal microbiota metabolites. The results will provide theoretical and technical support for the application of low-protein diets for the duck industry.

MATERIALS AND METHODS

Ethics Statement

All the procedures used in the study were approved by the Institutional Animal Care and Use Committee of Sichuan Agricultural University (SAUPN-19-02).

Experimental Design and Procedure

A total of 576 1-day-old male Pekin ducklings were obtained from a local hatchery and fed with the same standard starter diet containing 11.70 MJ/kg of ME, 19.5% CP, 1.1% lysine, 0.45% Met, 0.78% threonine, and 0.22% tryptophan until d 14. On d 14, they were randomly assigned to 36 cage pens, each with 16 ducks and having similar initial BW. All ducks were housed in an environmentally controlled facility. Ducks were provided with water and feed ad libitum throughout the experiment.

A 3 × 2 factorial arrangement was used with 3 dietary CP levels (17.5%, 15.5%, and 13.5%), with or without protease supplementation (20,000U/kg as per the manufacturer's recommendation), a total of 6 treatments with 6 replicates of 16 ducks each. Six isocaloric diets were formulated. Diets were fortified with synthetic feed-grade lysine, Met, threonine, and tryptophan to provide the Pekin ducks with recommended levels of

Table 1. Composition and nutrient contents of the experimental diets (DM basis) %.

	Dietary CP levels, %		
Items	13.5	15.5	17.5
Ingredients, %			
Corn	59.8	50.0	40.2
Cottonseed meal	4.00	6.00	8.00
Rapeseed meal	4.50	5.25	6.00
Wheat middlings	8.00	10.0	12.0
Rice bran	14.0	13.5	13.0
Feather meal	0.20	0.90	1.60
Distillers dried grains with solubles	4.00	8.00	12.0
Soybean oil	0.30	1.45	2.60
Calcium carbonate	1.22	1.26	1.29
Dicalcium phosphate	1.16	1.03	0.90
L-Lysine.HCl	0.82	0.74	0.66
DL-Methionine	0.32	0.29	0.26
L-Threonine	0.50	0.425	0.35
L-Tryptophan	0.10	0.08	0.06
Sodium chloride	0.35	0.35	0.35
Choline chloride (50%)	0.20	0.20	0.20
Vitamin and mineral premix[1]	0.53	0.53	0.53
Total	100.0	100.0	100.0
Calculated nutrients levels			
ME, MJ/kg	11.9	11.9	11.9
Calcium, %	0.79	0.79	0.79
Available phosphorus, %	0.35	0.35	0.35
Lysine, %	1.10	1.10	1.10
Methionine, %	0.53	0.53	0.53
Analyzed nutrients content			
CP, %	13.24	15.26	17.27
Total lysine, g/kg	0.94	1.07	0.99
Total methionine, g/kg	0.37	0.36	0.33
Total threonine, g/kg	0.73	0.83	0.75
Total valine, g/kg	0.55	0.63	0.61
Total leucine, g/kg	1.28	1.47	1.34
Total isoleucine, g/kg	0.40	0.45	0.40
Total histidine, g/kg	0.28	0.32	0.36
Total arginine, g/kg	0.81	1.04	1.07
Total essential amino acid, g/kg	5.87	6.77	6.44
Total phenylalanine, g/kg	0.51	0.61	0.60
Total glycine, g/kg	0.51	0.58	0.64
Total serine, g/kg	0.50	0.65	0.68
Total glutamic acid, g/kg	2.22	2.66	2.81
Total aspartic acid, g/kg	0.88	1.10	1.11
Total alanine, g/kg	0.54	0.70	0.69
Total cysteine, g/kg	0.25	0.25	0.21
Total proline, g/kg	0.85	1.00	0.97
Total tyrosine, g/kg	0.40	0.44	0.34
Total nonessential amino acids, g/kg	6.16	7.37	7.44

[1]Vitamin and mineral premix provides the following per kg of final diet: vitamin A, 8,000 IU; vitamin D_3, 2,000 IU; vitamin E, 5 mg; vitamin K_2, 1 mg; vitamin B_1, 0.6 mg; vitamin B_2, 4.8 mg; vitamin B_6, 1.8 mg; vitamin B_{12}, 0.009 mg; niacin, 10.5 mg; DL-calcium pantothenate, 7.5 mg; folic acid, 0.15 mg; Fe ($FeSO_4 \cdot H_2O$), 80 mg; Cu ($CuSO_4 \cdot 5H_2O$), 8 mg; Mn ($MnSO_4 \cdot H_2O$), 70 mg; Zn ($ZnSO_4 \cdot H_2O$), 90 mg; I (KI), 0.4 mg; Se (Na_2SeO_3), 0.3 mg.

Table 2. The primers used for quantitative real-time PCR.

Genes	Primer sequence, sense/antisense	Length (bp)	Gene ID
IL-6	Forward: CTGCGAGAACAGCATGGAGA Reverse: GAAAGGTGAAAAGCCCGCTG	191	XM_013100522
IL-10	Forward: GCTGGAGATGATGCGGTTCT Reverse: CACGTGAGGAACCTGTGACA	179	XM_013092231
TNF-α	Forward: ACCCCGTTACAGTTCAGACG Reverse: TAGCCATGTCAATGCTCCTG	140	XM_005027491.3
NF-κb	Forward: GAGCGTTTTCAAGAGGTTGC Reverse: AGGGATCTTCTCCTGCCATT	106	XM_005017679.4
IFN-γ	Forward: ACTGGCTTGAAAATCCAACG Reverse: GGAGACTGGCTCCTTTTCCT	101	NM_001310417.1
MUC2	Forward: ACTAGCACGAGGGAAGTGGA Reverse: TGGGATGTTGCAATGAGTGT	108	XM_005024513.3
ZO-1	Forward: TACGCCTGTGAAGAATGCAG Reverse: GGAGTGTGTGGTGTTTGCTTT	86	XM 013104939.1
Claudin-1	Forward: TCATGGTATGGCAACAGAGTGG Reverse: CGGGTGGGTGGATAGAAG	179	XM 013108556.1
Occludin	Forward: CAGGATGTGGCAGAGGAATACAA Reverse: CCTTGTCGTAGTCGCTCACCAT	160	XM 013109403.1
β-actin	Forward: AAGTACCCCATTGAACACGGT Reverse: TCTGTTGGCTTTGGGGTTCA	147	NM-0.013,10,421.1

Abbreviations: IFN-γ, interferon gamma; MUC-2, mucin 2; NF-κb, nuclear transcription factor; TNF-α, tumor necrosis factor α; ZO-1, Zonula occludens-1.

AA as per the NRC (1994). The composition of diets and the analyzed nitrogen content and AA composition of the experimental diets are presented in Table 1. The sources, traits, and supplemented dose of protease were the same as those of our previous study by Wang et al. (2020).

Data Collection and Measurement

On d 35, after withdrawing feed for 12 h, the ducks were weighed, and feed consumption was obtained from each pen. The feed intake (**FI**), BW gain, and feed-to-gain ratio were determined, mortality was recorded, and the dead birds' weights were used to adjust the feed-to-gain ratio.

Later, one of them with an average weight was selected from each replicate for blood collection (n = 6). Blood (approximately 6 mL) was collected via the vena brachialis and placed into the tubes with or without heparin sodium (an anticoagulant), and then, plasma and serum were isolated, respectively, for subsequent determination of blood parameters.

The ducks were finally euthanized by cervical dislocation after collecting the blood. Immediately after being euthanized, the small intestine and cecum were removed. The relative weight (g/100 g BW) and length (cm/100 g BW) of the intestinal segments, including the duodenum (from the gizzard to the bile duct), jejunum (from the bile duct to Meckel's diverticulum), and ileum (from Meckel's diverticulum to the ileocecal junction), were measured.

Tissue samples were excised from the middle of the ileum for morphologic measurement. After dissecting the ileum segment and removing about 2 g of the intestinal, the mucosal samples were scratched from the middle ileum (approximately 0.6 g) and collected into 2-mL microtubes to analyze the abundance of mRNA coding for inflammatory factors and barrier function genes. Finally, the cecal digesta was collected and frozen at −80 °C to measure the concentration of SCFA and BCFA.

Determination of Blood and Intestinal Parameters

The coagulated and the anticoagulated blood samples were centrifuged at 3,000 × *g* for 15 min at 4 °C to collect serum and plasma, respectively, and then stored at –80°C until further AA and inflammatory cytokine concentrations were analyzed. Serum AA concentration was determined by ion-exchange chromatography with an L8800 high-speed AA analyzer (Hitachi, Tokyo). The endotoxin (**ET**), IL-6, and tumor necrosis factor-α (**TNF-α**) in the plasma and ileum mucosal were determined spectrophotometrically using ELISA kits purchased from Sophia Biotechnology Co., Ltd. (MEIMIAN, Jiangsu, China) as per the manufacturer's instructions.

Histologic Examination of the Ileum

Formalin-fixed (10%) samples were prepared using the paraffin embedding procedures. Samples were sectioned at 5-μm size and stained with hematoxylin and eosin. A total of 20 intact, well-oriented crypt–villi units per sample were randomly selected and measured. The villus height (**VH**, from the tip of the villus to the crypt opening) and crypt depth (**CD**, from the base of the crypt to the level of the crypt opening) were determined using an image processing and analyzing system (Inverted microscope: NIKON CI-S, Tokyo, Japan; Imaging system: NIKON DS-U3, Tokyo, Japan).

RNA Isolation and Quantitative Real-Time PCR

The abundance of the mRNAs that encode *IL-6, IL-10, TNF-α, nuclear transcription factor-κb, interferon*

gamma, mucin 2, Zonula occludens-1, Claudin-1, and *Occludin* were determined as described previously (Bai et al., 2019). Briefly, total RNA was extracted using TRIzol reagent (TaKaRa, Dalian, Liaoning, China) following the manufacturer's instructions. RNA integrity was tested using 1% agarose gel electrophoresis. The RNA concentration was quantified using a spectrophotometer (Nano Drop 2000; Thermo Fisher Scientific Inc.). After determining the RNA concentration, 1 μg of total RNA was immediately reverse transcribed into cDNA using the Prime Script RT Reagent Kit (TaKaRa Biotechnology, Dalian, China) as per the manufacturer's instructions. Then, real-time PCR was performed in triplicate on an ABI 7900HT real-time PCR detection system (Applied Biosystems, CA) as per the manufacturer's instructions. The primer sequences for the target genes and β-actin are listed in Table 2. The relative levels of mRNA expression were calculated using the $2^{-\Delta\Delta CT}$method after normalization against the β-actin gene.

The Cecal SCFA and BCFA Assays

The cecal SCFA and BCFA concentration were determined as previously described (Qin et al., 2019). Approximately 0.5 g of cecal content was diluted with 2 mL of ultrapure water, mixed uniformly, and allowed to stand for 30 min and then centrifuged at 3,000 × *g* for 15 min. Supernatants (1 mL) were mixed with 0.2 mL of ice-cold 25% (w/v) metaphosphoric acid solution at 4 °C and incubated for 30 min, followed by centrifugation at 11,000 × *g* for 10 min. The cecal SCFA and BCFA, including acetate, propionate, butyrate, isobutyrate, isovalerate, and valerate, were separated and determined by gas chromatography (Varian CP-3800).

Statistical Analysis

All data were analyzed by 2-way ANOVA using the GLM procedure of SAS software (version 9.2; SAS Institute Inc., Cary, NC). This model included the main effects of dietary CP levels, dietary protease supplementation, and their interaction. The pen was considered as an experimental unit. The means showing significant treatment differences at $P < 0.05$ in ANOVA were then compared with Fisher's least significant difference procedure, and an alpha level of 0.05 was considered significant. All the data were tested for normality by the UNIVARIATE procedure and common variance using the GLM procedure.

RESULTS

Growth Performance

As shown in Table 3, the dietary CP levels × protease interaction was not significant ($P > 0.05$) for the growth performance of ducks between 15 and 35 d. Reduced dietary CP levels (-2% or -4%) had a significant negative effect ($P < 0.05$) on the growth performance and FI of ducks. The more the dietary CP levels were reduced, the more the growth performance was decreased.

Serum AA Profile

The dietary CP levels × protease interaction was significant ($P < 0.05$; Table 4 and Table 5) for serum-free Arg concentration. Supplemented protease in a diet containing 13.5% CP significantly decreased the serum-free Arg concentration compared with the same diet without protease. Irrespective of protease supplementation, low-protein diets (13.5% CP) had lower ($P < 0.05$) serum-free Arg, Ile, Leu, Met, Phe, Val, and Pro but a higher serum-free glycine concentration than those found in the normal-protein diet (17.5% CP). Similarly, irrespective of dietary CP levels, protease supplementation markedly increased ($P < 0.05$) the serum-free glutamic acid (**Glu**) concentration.

Plasma Inflammatory Cytokine Concentration

As shown in Table 6, the dietary CP levels × protease interaction was significant ($P < 0.05$) for plasma IL-6 and TNF-α concentration. Supplementing protease in a diet containing 17.5% CP significantly decreased ($P < 0.05$) the serum IL-6 concentration when compared with the same diet without protease. However, the supplemented protease in a diet with 13.5% CP significantly increased ($P < 0.05$) the serum TNF-α concentration compared with the same diet without protease.

Diets with 13.5% CP had a higher ($P < 0.05$) plasma IL-6 and TNF-α concentration than those in the diets with 15.5% or 17.5% CP. Similarly, ducks fed with a diet containing 15.5% CP had a higher ($P < 0.05$) plasma TNF-α concentration than the ducks fed 17.5% CP. Irrespective of the dietary CP levels, protease supplementation significantly decreased ($P < 0.05$) the plasma ET and IL-6 levels, while increased ($P < 0.05$) the plasma TNF-α concentration.

Intestinal Development and Morphology

As shown in Table 7 and 8, ducks fed with a diet containing 13.5% CP had higher ($P < 0.05$) relative length of the duodenum, jejunum, and ileum and lower ileal VH than ducks fed 17.5% CP. A significant interaction ($P < 0.05$; Table 8) between dietary CP levels and protease supplementation was observed for VH:CD. The supplemented protease in the diet containing 13.5% CP significantly decreased ($P < 0.05$) the VH:CD of the ileum compared with the same diet without protease.

Ileal Mucosal Cytokines Concentration and Cytokine Genes Expression

The dietary CP levels × protease interaction was significant ($P < 0.05$; Table 9) for ileal mucosal IL-6 concentration. Supplemented protease in the diet containing 13.5% CP significantly increased the ileal

Table 3. Effects of low-protein diets and protease supplementation on growth performance of ducks from 15 to 35 d of age.[1]

CP%	Protease supplementation	14 d BW(g)	35 d BW(g)	15–35 d BWG (g)	15–35 d FI (g)	15–35 d F:G
13.5	-	749.5	1,636	886.3	2,259	2.64
15.5	-	747.3	2,097	1,350	3,129	2.32
17.5	-	743.7	2,621	1,877	3,855	2.05
13.5	+	744.4	1,662	918.0	2,369	2.59
15.5	+	748.2	2,086	1,337	3,065	2.30
17.5	+	749.0	2,485	1,736	3,771	2.17
	SEM	6.93	59.06	59.40	94.90	0.09
Main effect						
CP%	13.5	746.9	1,649^{c}	902^{c}	2,314^{c}	2.61^{a}
	15.5	747.8	2,092^{b}	1,344^{b}	3,097^{b}	2.31^{b}
	17.5	746.3	2,553^{a}	1,807^{a}	3,813^{a}	2.11^{c}
	SEM	4.90	41.76	42.00	67.10	0.06
Protease	-	746.8	2,118	1,371	3,081	2.34
	+	747.2	2,078	1,330	3,068	2.35
	SEM	4.00	34.10	34.30	54.79	0.05
Source of variation		Probability				
P-value	CP	0.978	<0.001	<0.001	<0.001	<0.001
	Protease	0.947	0.408	0.407	0.869	0.843
	CP*protease	0.754	0.368	0.332	0.541	0.572

[a–c]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviation: F:G, feed-to-gain ratio.
[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

Table 4. Effects of low-protein diets and protease supplementation on serum-free essential amino acid concentrations (mmol/L) of ducks at 35 d of age.[1]

CP%	Protease supplementation	Arg	His	Ile	Lys	Leu	Met	Phe	Val	Thr
13.5	-	0.16^{a,b}	0.09	0.06	0.15	0.16	0.03	0.16	0.13	0.58
15.5	-	0.19^{a,b}	0.11	0.08	0.19	0.19	0.04	0.19	0.20	0.50
17.5	-	0.17^{a,b}	0.11	0.10	0.23	0.23	0.06	0.22	0.25	0.63
13.5	+	0.07^{c}	0.11	0.05	0.15	0.16	0.03	0.16	0.11	0.65
15.5	+	0.13^{b,c}	0.10	0.06	0.16	0.15	0.04	0.18	0.13	0.63
17.5	+	0.22^{a}	0.11	0.09	0.20	0.23	0.08	0.23	0.24	0.68
	SEM	0.02	0.01	0.01	0.03	0.02	0.01	0.02	0.03	0.09
Main effect										
CP%	13.5	0.12^{b}	0.10	0.06^{b}	0.15	0.16^{b}	0.03^{b}	0.16^{b}	0.12^{b}	0.62
	15.5	0.16^{a,b}	0.10	0.07^{b}	0.17	0.17^{b}	0.04^{b}	0.18^{b}	0.16^{b}	0.57
	17.5	0.19^{a}	0.11	0.09^{a}	0.21	0.23^{a}	0.07^{a}	0.23^{a}	0.25^{a}	0.66
	SEM	0.02	0.01	0.01	0.02	0.02	0.01	0.02	0.02	0.06
Protease	-	0.17	0.10	0.08	0.19	0.19	0.04	0.19	0.19	0.57
	+	0.14	0.10	0.07	0.17	0.18	0.05	0.19	0.16	0.66
	SEM	0.01	0.01	0.01	0.02	0.01	0.00	0.01	0.01	0.05
Source of variation		Probability								
P-value	CP	<0.01	0.490	<0.01	0.085	<0.05	<0.001	<0.05	<0.001	0.609
	Protease	0.082	0.673	0.292	0.376	0.479	0.512	0.882	0.143	0.248
	CP*protease	<0.05	0.321	0.561	0.816	0.637	0.705	0.941	0.470	0.891

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviations: Arg, arginine; His, histidine; Ile, isoleucine; Lys, lysine; Leu, leucine; Met, methionine; Phe, phenylalanine; Val, valine; Thr, threonine.
[1]Values are the means of 6 ducks per treatment (n = 6).

Table 5. Effects of low-protein diets and protease supplementation on serum free nonessential amino acid concentrations (nmol/L) of ducks at 35 d of age.[1]

CP%	Protease supplementation	Ala	Asp	Cys	Gly	Glu	Pro	Tyr	Ser
13.5	-	0.94	0.07	0.04	1.43	0.13	0.27	0.13	0.59
15.5	-	1.02	0.06	0.04	1.47	0.12	0.33	0.13	0.56
17.5	-	1.06	0.05	0.05	1.15	0.10	0.46	0.16	0.59
13.5	+	1.17	0.06	0.05	1.45	0.13	0.34	0.18	0.85
15.5	+	1.18	0.07	0.06	1.30	0.15	0.33	0.19	0.64
17.5	+	1.06	0.05	0.06	1.13	0.13	0.38	0.16	0.53
	SEM	0.15	0.01	0.01	0.12	0.01	0.04	0.03	0.09
Main effect									
CP%	13.5	1.06	0.07	0.04	1.44[a]	0.13	0.31[b]	0.15	0.72
	15.5	1.10	0.07	0.05	1.39[a]	0.14	0.33[a,b]	0.16	0.60
	17.5	1.06	0.05	0.06	1.14[b]	0.11	0.42[a]	0.16	0.56
	SEM	0.11	0.01	0.01	0.08	0.01	0.03	0.02	0.06
Protease	-	1.01	0.06	0.04	1.35	0.11	0.35	0.14	0.58
	+	1.14	0.06	0.06	1.29	0.14	0.35	0.18	0.67
	SEM	0.09	0.00	0.00	0.07	0.01	0.03	0.01	0.05
Source of variation		Probability							
P-value	CP	0.954	0.126	0.272	<0.05	0.122	<0.05	0.905	0.200
	Protease	0.297	0.948	0.082	0.552	<0.05	0.914	0.075	0.207
	CP*protease	0.746	0.548	0.560	0.702	0.216	0.272	0.376	0.237

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviations: Ala, alanine; Asp, aspartic acid; Cys, cysteine; Gly, glycine; Glu, glutamic acid; Pro, proline; Tyr, tyrosine; Ser, serine.
[1]Values are the means of 6 ducks per treatment (n = 6).

mucosal IL-6 concentration compared with the same diet without protease. Regardless of the protease addition, ducks fed with a diet containing 15.5% CP had a higher ($P < 0.05$) ET concentration of ileal mucosa than ducks fed 13.5% CP. Feeding the ducks with a normal-protein diet (17.5% CP) decreased ($P < 0.05$) the TNF-α concentration in ileal mucosa compared with diets with 13.5% or 15.5% CP.

As shown in Table 10, ducks fed with a low-protein diet (13.5% CP) had lower ($P < 0.05$) mRNA expression of *TNF-α, nuclear transcription factor-κb, and interferon gamma* in ileal mucosa than the ducks fed diets with 17.5% CP.

Expression of Ileal Barrier–Related Genes

As shown in Table 11, low-protein diets (13.5% or 15.5%) significantly decreased ($P < 0.05$) the mRNA expression of *Occludin* in ileal mucosa compared with the diet with 17.5% CP.

Cecal SCFA and BCFA

As shown in Table 12, the dietary CP levels × protease interaction was significant ($P < 0.05$) for the cecal acetate and total SCFA concentration. Supplemented protease in a diet containing 13.5% CP significantly decreased the cecal acetate concentration compared with the same diet without protease. Reduced dietary CP levels (-2% or –4%) significantly decreased ($P < 0.05$) the cecal acetate, propionate, and total SCFA concentration in ducks compared with the diet containing 17.5% CP. Irrespective of the dietary CP level, protease supplementation could significantly decrease ($P < 0.05$) the cecal isovalerate and total BCFA concentration.

DISCUSSION

It is well-documented that lowering dietary CP level reduces the performance of poultry (Alleman and Leclercq, 1997), which was further validated by our data, where low-protein diets (13.5% and 15.5%) could suppress the BW, BW gain, and FI of ducks aged between 15 to 35 d; and, the more the dietary CP levels were reduced, the more the growth performance of Pekin ducks was decreased. These findings are consistent with those of the previous studies, where irrespective of the dietary ME, the ducks fed with 15% CP diets had lower BW and BW gain compared with those fed with 17% and 19% CP diets (Zeng et al., 2015); and, feeding the ducks, aged between 15 d to 49 d, with 15.5%, 14.5% and 13.5% CP resulted in poor growth performance compared with those fed with 17.5% and 16.5% CP diets (Wang et al., 2020). The reason for the inhibition of growth performance and FI of reducing protein diets in this current experiment may be that the addition of low digestibility of feedstuffs, such as rapeseed, cottonseed and feather, decreased intestinal health to some extent.

Table 6. Effects of low-protein diets and protease supplementation on plasma inflammation cytokines content of ducks at 3 5d of age.[1]

CP%	Protease supplementation	Endotoxin (ng/L)	IL-6 (Ng/L)	TNF-α (Ng/L)	Urea (mmol/L)
13.5	-	180.9	38.35[a]	454.4[b]	0.34
15.5	-	177.9	31.71[b]	555.0[a]	0.37
17.5	-	180.2	35.33[a]	426.5[b]	0.39
13.5	+	168.7	38.79[a]	593.5[a]	0.35
15.5	+	162.3	31.20[b]	592.9[a]	0.34
17.5	+	167.5	29.04[b]	415.1[b]	0.42
	SEM	4.78	1.14	16.54	0.03
Main effect					
CP%	13.5	174.8	38.57[a]	523.9[b]	0.34
	15.5	170.1	31.46[b]	574.0[a]	0.35
	17.5	173.8	32.18[b]	420.8[c]	0.41
	SEM	3.38	0.81	11.69	0.02
Protease	-	179.7	35.13	478.7	0.36
	+	166.2	33.01	533.8	0.38
	SEM	2.76	0.66	9.55	0.01
Source of variation		Probability			
P-value	CP	0.591	<0.001	<0.001	0.246
	Protease	<0.01	<0.05	<0.001	0.280
	CP*protease	0.926	<0.05	<0.001	0.618

[a–c]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviation: *TNF-α*, tumor necrosis factor-α.
[1]Values are the means of 6 ducks per treatment (n = 6).

Table 7. Effects of low-protein diets and protease supplementation on intestinal development of ducks from 14 to 35 d of age.[1]

		Relative length (cm/100g of live BW)			Relative weight (g/100g of live BW)		
CP%	Protease supplementation	Duodenum	Jejunum	Ileum	Duodenum	Jejunum	Ileum
13.5	-	1.95	4.44	4.34	0.29	0.63	0.66
15.5	-	1.52	3.80	3.58	0.30	0.70	0.62
17.5	-	1.36	3.15	2.51	0.27	0.62	0.47
13.5	+	1.89	4.25	4.17	0.30	0.69	0.62
15.5	+	1.55	3.81	3.77	0.24	0.60	0.56
17.5	+	1.44	3.15	3.10	0.30	0.58	0.63
	SEM	0.101	0.194	0.208	0.021	0.055	0.056
Main effect							
CP%	13.5	1.92[a]	4.35[a]	4.26[a]	0.29	0.66	0.64
	15.5	1.53[b]	3.80[b]	3.68[b]	0.27	0.65	0.59
	17.5	1.40[b]	3.15[c]	2.80[c]	0.29	0.60	0.55
	SEM	0.071	0.137	0.147	0.015	0.039	0.039
Protease	-	1.61	3.80	3.48	0.29	0.65	0.58
	+	1.62	3.74	3.68	0.28	0.63	0.61
	SEM	0.058	0.112	0.120	0.012	0.032	0.032
Source of variation				Probability			
P-value	CP	<0.001	<0.001	<0.001	0.465	0.546	0.287
	Protease	0.859	0.707	0.250	0.743	0.616	0.604
	CP*protease	0.796	0.836	0.204	0.136	0.346	0.142

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Values are the means of 6 ducks per treatment (n = 6).

Table 8. Effects of low-protein diets and protease supplementation on ileal morphology of ducks at 35 d of age.[1]

CP%	Protease supplementation	Villus height (μm)	Crypt depth(μm)	VH:CD
13.5	-	548.5	124.6	4.47[a]
15.5	-	629.1	176.0	3.61[b]
17.5	-	585.8	145.3	4.08[a,b]
13.5	+	507.2	135.3	3.76[b]
15.5	+	614.3	150.2	4.10[a,b]
17.5	+	649.9	143.1	4.55[a]
	SEM	34.47	9.76	0.16
Main effect				
CP%	13.5	527.9[b]	129.9[b]	4.11[a,b]
	15.5	621.7[a]	163.1[a]	3.86[b]
	17.5	617.8[a]	144.2[a,b]	4.32[a]
	SEM	24.38	6.90	0.11
Protease	-	587.8	148.7	4.05
	+	590.5	142.8	4.14
	SEM	19.90	5.63	0.09
Source of variation		Probability		
P-value	CP	<0.05	<0.01	<0.05
	Protease	0.923	0.469	0.515
	CP*protease	0.297	0.183	<0.001

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviations: CD, crypt depth; VH, villus height.
[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

Table 9. Effects of low-protein diets and protease supplementation on inflammation cytokines content of ileal mucosa of ducks at 35 d of age.[1]

CP%	Protease supplementation	Endotoxin (ng/L)	IL-6 (ng/L)	TNF-α (ng/L)
13.5	-	180.8	34.83^{b}	558.2
15.5	-	203.0	38.26^{a,b}	461.2
17.5	-	194.7	36.59^{a,b}	420.3
13.5	+	162.0	41.56^{a}	496.7
15.5	+	202.1	35.20^{b}	534.5
17.5	+	191.9	35.99^{b}	449.7
	SEM	8.74	1.76	27.39
Main effect				
CP%	13.5	171.4^{b}	38.20	527.5^{a}
	15.5	202.6^{a}	36.73	497.8^{a}
	17.5	193.3^{a,b}	36.29	435.0^{b}
	SEM	6.18	1.241	19.37
Protease	-	192.9	36.56	479.9
	+	185.4	37.58	493.7
	SEM	5.04	1.01	15.81
Source of variation		Probability		
P-value	CP	<0.005	0.530	<0.01
	Protease	0.301	0.481	0.543
	CP*protease	0.539	<0.05	0.057

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviation: *TNF-α*, tumor necrosis factor-α.
[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

Table 10. Effects of low-protein diets and protease supplementation on gene expression of inflammatory factors of ileal mucosa of ducks at 35 d of age.[1]

CP%	Protease supplementation	*IL-6*	*IL-10*	*TNF-α*	*NF-κb*	*Interferon gamma*
13.5	-	0.49	0.71	0.47	0.75	0.73
15.5	-	0.92	1.37	0.55	0.99	0.83
17.5	-	1.00	1.00	1.00	1.00	1.00
13.5	+	0.61	0.79	0.42	0.86	0.78
15.5	+	0.72	0.92	0.63	0.97	0.88
17.5	+	0.73	1.61	1.66	1.18	1.25
	SEM	0.13	0.28	0.21	0.11	0.12
Main effect						
CP%	13.5	0.55	0.75	0.45^{b}	0.80^{b}	0.76^{b}
	15.5	0.82	1.15	0.59^{b}	0.98^{a,b}	0.86^{b}
	17.5	0.86	1.30	1.33^{a}	1.09^{a}	1.13^{a}
	SEM	0.10	0.20	0.15	0.08	0.08
Protease	-	0.80	1.03	0.67	0.91	0.85
	+	0.68	1.10	0.91	1.00	0.97
	SEM	0.08	0.16	0.12	0.06	0.07
Source of variation			Probability			
P-value	CP	0.054	0.144	<0.001	<0.05	<0.05
	Protease	0.286	0.741	0.186	0.319	0.239
	CP*protease	0.321	0.187	0.219	0.635	0.612

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviations: NF-κb, nuclear transcription factor-κb; TNF-α, tumor necrosis factor-α.
[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

Table 11. Effects of low-protein diets and protease supplementation on gene expression of ileal barrier function of ducks at 35 d of age.[1]

CP%	Protease	*MCU2*	*ZO-1*	*Claudin-1*	*Occludin*
13.50	-	0.87	0.79	0.43	0.52
15.50	-	0.66	0.59	0.46	0.51
17.50	-	1.00	1.00	1.00	1.00
13.50	+	0.79	0.68	0.92	0.54
15.50	+	1.03	0.91	0.69	0.73
17.50	+	0.75	0.58	1.48	0.72
	SEM	0.18	0.15	0.35	0.11
Main effect					
CP%	13.50	0.83	0.74	0.68	0.53[b]
	15.50	0.84	0.75	0.58	0.62[b]
	17.50	0.88	0.79	1.24	0.86[a]
	SEM	0.13	0.11	0.25	0.08
Protease	-	0.84	0.79	0.63	0.68
	+	0.86	0.73	1.03	0.66
	SEM	0.10	0.09	0.20	0.07
Source of variation		Probability			
P-value	CP	0.967	0.932	0.146	<0.05
	Protease	0.936	0.605	0.176	0.867
	CP*protease	0.210	0.067	0.917	0.102

[a–b]Values within a column with no common superscripts differ significantly ($P < 0.05$).
Abbreviations: MUC2, mucin 2; ZO-1, Zonula occludens.
[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

The development and morphologic parameters, including VH, CD, and/or VH:CD, measured at the intestinal level, are widely used as a standard to evaluate the intestinal health of poultry (Ducatelle et al., 2018). In this study, we observed that low-protein diets (13.5% or 15.5% CP) damaged ileal morphology and decreased serum-free EAA, especially the free branched-chain AA concentration, and this damage depends on the reduced CP levels in the diet. Similarly, a less than optimal quality and concentration of protein can adversely impact the intestinal development and function (Gilbert et al., 2008; Wijtten et al., 2010). When the CP level decreased, the VH and VH:CD of the duodenum, jejunum, and ileum were significantly reduced in broilers (Ding et al., 2016). Macelline et al. (2019) also found that broilers fed with low-protein diets (18% CP) had decreased VH and CD compared with those fed with high-protein diets (23.3% CP). Law et al. (2018) found that broilers fed with low-protein diets (i.e., 17.2 and 15.6% CP in the starter and finisher diets, respectively) supplemented with synthetic AA, on d 35, displayed poorer intestinal architecture compared with the broilers fed with high-protein diets (21% and 19% CP in starter and finisher diets, respectively).

Many studies have suggested that the adverse effect of low-protein diets on intestinal architecture can be attributed to the reduction in nonessential AA (**NEAA**) level, such as glycine, Glu, and Pro, which are necessary to develop the gut epithelium and produce digestive secretions and mucin (Law et al., 2018). These reports were further validated by our data, which shows that low-protein diets (13.5% CP) had lower dietary EAA (5.87 g/kg) and NEAA (6.16 g/kg) concentration than the diets containing 17.5% CP (6.44 g/kg EAA; 7.44 g/kg NEAA). Consistent with the previous studies, we also found that serum-free Arg, Ile, Leu, Met, Phe, Val, and Pro concentration decreased in the ducks fed with low-protein diets (13.5% CP). However, in our previous study by Wang et al. (2020), we found that low-protein diets (13.5% CP) had the same SID of AA as that of the 17.5% CP diets. These results suggest that most of the AA undergo catabolism in the intestinal mucosa of ducks fed with low-protein diets. Zhang et al. (2013) found that serum concentrations of branched-chain AA (Ile, Leu, and Val) were reduced in piglets fed with a low-protein diet, although the branched-chain AA values were similar to those of the normal protein group. Yin et al. (2019) also found that glutamic-oxaloacetic transaminase activity, which indicates the Glu catabolism extent in the ileum, was higher in broilers fed with low-protein diets. These results indicate that low-protein diets increase the catabolism of intestinal mucosal AA to maintain the intestinal architecture and function of the poultry.

Significantly, we found that low-protein diets increased the system and local ileal inflammatory response, changed the ileal barrier function, and decreased the cecal total SCFAs concentration. The underlying reason may be not only the dietary CP levels

Table 12. Effects of low-protein diets and protease supplementation on cecal short-chain fatty acids and branch-chain fatty acids content of ducks at 35 d of age.[1]

		mmol/g							
CP%	Protease supplementation	Acetate	Propionate	Butyrate	Isobutyrate	Isovalerate	Valerate	SCFA	BCFA
13.5	-	37.8^{a,b}	7.53	3.59	0.33	1.02	1.99	48.92^{b}	3.34
15.5	-	36.1^{b}	8.90	4.18	0.31	0.92	2.02	49.19^{b}	3.25
17.5	-	39.2^{a,b}	13.0	4.16	0.42	1.07	1.81	56.39^{b}	3.30
13.5	+	15.1^{c}	4.19	2.41	0.18	0.47	1.53	17.51^{c}	1.81
15.5	+	24.1^{b,c}	7.52	3.31	0.38	0.60	1.29	34.96^{b,c}	2.27
17.5	+	55.9^{a}	18.7	5.24	0.27	0.63	2.20	82.79^{a}	3.10
	SEM	6.19	2.39	1.09	0.06	0.17	0.40	8.86	0.42
Main effect									
CP%	13.5	27.5^{b}	6.01^{b}	3.30	0.26	0.77	1.78	33.21^{b}	2.58
	15.5	30.1^{b}	8.21^{b}	3.74	0.35	0.76	1.65	42.07^{b}	2.76
	17.5	47.5^{a}	15.9^{a}	4.52	0.35	0.85	2.00	69.59^{a}	3.20
	SEM	4.38	1.62	0.67	0.04	0.11	0.27	6.27	0.30
Protease	-	37.7	9.81	3.98	0.35	1.00	1.94	51.50	3.30
	+	32.9	10.5	3.67	0.28	0.57	1.68	45.09	2.39
	SEM	3.69	1.30	0.63	0.04	0.09	0.21	5.12	0.24
Source of variation		Probability							
P-value	CP	<0.001	<0.001	0.260	0.203	0.784	0.630	<0.001	0.332
	Protease	0.251	0.855	0.675	0.123	<0.005	0.388	0.383	<0.05
	CP*protease	<0.05	0.117	0.448	0.120	0.774	0.301	<0.01	0.302

[a–c]Values within a column with no common superscripts differ significantly ($P < 0.05$).

Abbreviations: BCFA, branch-chain fatty acid; SCFA, short-chain fatty acid.

[1]Values are the means of 6 replicates of 16 ducks each (n = 6).

but also a lower digestibility of feed ingredients that were used in the experimental diets. Poorly digested diets, fasting, ET, and several other forms of stress have been shown to adversely affect the intestinal barrier function (Gilani et al., 2016). Dietary CP level and its digestibility affect the formation and quantity of microbial metabolites resulting from the hindgut protein fermentation. In weaned pigs fed with a corn–soybean–wheat diet, ammonia N in ileal digesta was reduced linearly as dietary CP was decreased, and with the exception of valeric acid, volatile fatty acid levels in ileal digesta of piglets fed low-protein diets were generally lower than in those fed the control diet (Nyachoti et al., 2006). These results showed that reducing dietary protein levels may lead to most protein being absorbed (including crystalline AA) by the small intestine, reducing the entry of undigested proteins into the cecum, thus inhibiting the growth of acidogenic bacteria, leading to the reduction in SCFA.

However, SCFA shape the gut environment and maintain the intestinal barrier function, especially butyrate, which can fuel the intestinal epithelial cells and improve intestinal integrity. The increased butyrate production may be associated with improved gut barrier function by mitigating the inflammation, enhancing tight junctions, and accumulating mucus (Segain et al., 2000; Peng et al., 2009; Bach Knudsen et al., 2018). Correspondingly, we found that low-protein diets increased plasma IL-6 and TNF-α levels along with the ileal mucosal TNF-α concentration while downregulating the mRNA expression of *Occludin* in ileal mucosa. Tight junctions act as a critical barrier in the epithelial defense that protects the birds from translocating pathogens and allergens and maintain their productivity (Ballard et al., 1995). The studies of Ulluwishewa et al. (2011) and Li et al. (2018) demonstrated that weakening of tight junctions increased the intestinal permeability leading to elevated serum ET levels. When ET enters the bloodstream, it activates Toll-like receptor 4 that is located at the surface of immune cells, leading to the release of proinflammatory cytokines, such as IL-6 and TNF-α (Nguyen et al., 2014). These results suggest that reducing CP levels (-4%) damage gut health to induce systemic inflammatory response, which is a serious stress response to ducks.

Interestingly, our study also revealed that protease supplementation increased the serum-free Glu levels while decreasing the concentration of serum-free Arg, plasma ET and IL-6, and cecal isovalerate. A large proportion of AA is absorbed along the small intestine and fails to enter the portal circulation, thus becoming available for protein accretion because of their extensive utilization in the gut mucosa via anabolic and catabolic pathways (Stoll et al., 1998). Glutamic acid is the most catabolized AA to provide energy in the gut mucosa (Yin et al., 2019). A significant increase in protease supplementation may release more Glu to mitigate the catabolism of EAA. Arg is an EAA for poultry and can be metabolized to produce important molecules, such as nitric oxide, polyamines, and creatine. Zhang et al. (2017) demonstrated that the addition of dietary Arg protects the gut mucosa by improving the innate immune response, intestinal absorption, and the barrier function through suppressing the colonization of *Clostridium perfringens* in the necrotic enteritis–challenged broiler chickens. A significant decrease in serum-free Arg concentration in low-protein diets supplemented with protease, indicates that protease addition can increase the Arg metabolism in the gastrointestinal tract to maintain intestinal health. At the same time, the formation of the BCFA, including isobutyrate, 2-methyl butyrate, and isovalerate in the cecum, occurred from the degradation of Val, Ile, and Leu in the intestinal tract of animals (Qaisrani et al., 2015). The significant decrease also indicated that protease supplementation could increase the EAA utilization in the upper gastrointestinal tract to maintain intestinal integrity. This is why protease supplementation can reduce plasma ET and IL-6 concentration in the present study. Park et al. (2020) also observed that protease added to soybean meal–based diets could improve gut health to reduce serum concentration of proinflammatory cytokines in weanling pigs.

CONCLUSION

In summary, low-protein diets (13.5% or 15.5% CP) with lower digestibility of feed ingredients suppressed the growth performance and feed intake by increasing the catabolism of intestinal mucosal AA and decreasing cecal SCFA production to damage gut health, such as impaired ileal architecture and barrier function and increased ileal mucosal inflammation. Ducks fed diets with 13.5% CP presented a serious systemic inflammatory response. The more the dietary CP levels were reduced, the more the growth performance was decreased and more serious was the intestinal mucosal damage. Protease supplementation can attenuate intestinal mucosal injury via increasing the metabolism of EAA or NEAA in the gastrointestinal tract to improve intestinal integrity. Using low-protein diets in the poultry industry will need more concern for the intestinal health and N or AA metabolism of the gut microbiome.

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DISCLOSURES

The authors declare no conflicts of interest.

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