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The effects of low-protein diets and protease supplementation on broiler chickens in a hot and humid tropical environment

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Objective: This experiment was conducted to investigate the effects of dietary crude protein (CP) level and exogenous protease supplementation on growth performance, serum metabolites, carcass traits, small intestinal morphology and endogenous protease activity in broiler chickens reared under a tropical climate.
Methods: A total of 480 day-old male broiler chicks were randomly assigned to eight dietary treatments in a 4×2 factorial arrangement. The main effects were CP level (21.0%, 19.7%, 18.5%, or 17.2% from 1 to 21 days and 19.0%, 17.9%, 16.7%, or 15.6% from 22 to 35 days) and protease enzyme supplementation (0 ppm or 500 ppm). All experimental diets were fortified with synthetic feed-grade lysine, methionine, threonine and tryptophan to provide the minimum amino acid recommended levels for Cobb 500.
Results: Reducing dietary CP linearly reduced ($p<0.05$) growth performance, serum albumin, total protein, and carcass traits and increased ($p<0.05$) serum triglycerides and abdominal fat. There was no consistent effect of reducing dietary CP on morphological parameters of the intestine and on the pancreatic and intestinal endogenous protease activity ($p>0.05$). Protease supplementation improved ($p<0.05$) feed conversion ratio, body weight gain, carcass yield and intestinal absorptive surface area.
Conclusion: Protease supplementation, as measured by growth performance, intestinal morphology and carcass yield, may alleviate the detrimental effects of low protein diets in broiler chickens.

Keywords: Broiler Chickens; Microbial Protease; Low-protein Diet; Growth Performance; Intestinal Morphology

INTRODUCTION

Cost minimization and environmental concern are consistently driving poultry nutritionists to improve the efficiency of feed utilization by formulating digestible, practical diets to avoid overfeeding with nutrients. Over the past few decades, numerous studies have been published on the use of synthetic amino acids (AAs) in low protein (LP) diets [1,2,3]. The supplementation of AAs in these studies varied widely depending on the type of supplemented AA, such as first and second limiting AA, feed-grade AAs, all essential amino acids (EAAs) or both EAAs and non-essential amino acids (NEAAs). However, usage of LP diets in some excessive levels still has a negative impact on growth performance, carcass traits [1], morphology of intestinal villi [4] and profitability. The scenario is more challenging when broilers are raised under high-temperature conditions, either during summer in temperate regions or in a tropical climate [5]. Although one study suggested LP diets as a means of reducing metabolic heat production associated with protein catabolism [6], others suggested the use of higher dietary crude protein (CP) mainly due to lower feed intake (FI) and protein digestibility under high-temperature conditions [7]. It is possible that the combination of

these two strategies results in better performance by providing an LP diet supplemented with commercially available feed-grade AA and exogenous enzymes [8-10].

Although use of exogenous enzyme blends (i.e., carbohydrases, phytase, and protease) is well-established and widely accepted to improve digestibility of nutrient elements [8,10], use of single proteases is still under investigation for widespread application. Single exogenous protease, when added to the diet, effectively increased the apparent AA and protein digestibility in the broiler chicken's diet, even in an LP diet [11,12]. However, some reports did not confirm the conversion of those improvements in broiler performance [13,14]. Proteases may have an 'extra-proteinaceous' advantage for broilers reared under high temperature conditions by improving gut health [15]. Supplemental mono-component protease reduced the population of intestinal clostridium [16] and improved intestinal villi height (VH) and VH/crypt depth (CD) ratio [17] in broiler chickens.

The health and development of the intestinal epithelium and its absorptive surface area (ASA) are reported to be diminished under heat stress conditions, leading to poorer nutrient absorption and performance in broiler chickens [18]. Thus, the current study was undertaken to explore the effect of exogenous microbial protease supplementation on growth performance, blood metabolites, carcass traits and intestinal morphology in broiler chickens that were fed LP diets and reared under a hot and humid tropical climate.

MATERIALS AND METHODS

Experimental design and diets

Four isocaloric diets with descending levels of CP namely i) 21.0% (day 1 to 21) and 19.0% (day 22 to 35), ii) 19.7% (day 1 to 21) and 17.9% (day 22 to 35), iii) 18.5% (day 1 to 21) and 16.7% (day 22 to 35), iv) 17.2% (day 1 to 21) and 15.6% (day 22 to 35) were formulated. Diets were fortified with synthetic feed-grade lysine, methionine, threonine and tryptophan to provide the minimum AA recommended levels for Cobb 500 (Table 1). Four hundred and eighty birds were randomly assigned to diets without or with microbial protease (300,000 units/kg) supplementation from day 1 to 35. Thus, the experiment had a 4×2 factorial arrangement of treatments with diets containing four levels of CP and two levels of enzyme supplementation. There were four replicate pens per dietary CP-enzyme supplementation subgroup.

Enzyme composition

A purified microbial protease (Cibenza DP100; Novus International Inc., St. Charles, MO, USA) was used in this study. The enzyme is an alkaline serine endopeptidase protease derived from *Bacillus lichenformis* with a protease activity of 600,000 units/g. The manufacturer's recommended inclusion rate is 300 units/g of feed. The protease activity was determined using the method described by Jin et al [19]. The protease activity in protease-added diets for NP, LP1, LP2, and LP3 diets were 324, 294, 325, and 327 units per gram of starter feed and 305, 325, 286, and 321 units per gram of finisher feed, respectively.

Birds and management

All experimental procedures were conducted in accordance with Universiti Putra Malaysia Research Policy on Animal Ethics and Welfare. A total of 480 day-old male Cobb 500 broiler chicks (46.56 g±0.01) were randomly distributed to 32 floor pens (n = 15 chicks/pen) with wood shavings that serve as deep litter material in a conventional open-sided poultry house and under the hot and humid tropical conditions. The temperature ranged from 23°C to 36°C, and the relative humidity was between 75% and 90%. Respective diets (mash feed) and water were provided *ad libitum* from day (d) 1 to 35.

Data collection and sampling

Birds and feed were weighed by pen group at the initial of the experiment (d 1) and the remaining of the feed and body weight were recorded on d 1, 21, and 35. Feed supple and left over of the feed consumption were weighed during the same period. The FI data was adjusted for mortalities and feed conversion ratio (FCR) was calculated as ratio of feed consumption to weight gain. Mortality was recorded as it occurred. On d 35, two birds per replicate were randomly selected, individually weighed and sacrificed for collection of blood, intestinal tissue and digesta. Blood samples were centrifuged at 3,000 g at 4°C for 15 min to obtain the serum. The haemolysis-free serum was transferred immediately and frozen at –80°C until further biochemical analyses. For measurement of intestinal morphology, approximately 5 cm of the middle portion of the duodenum (apex section), jejunum (between the entry of bile ducts and Meckel's diverticulum) and ileum (between Meckel's diverticulum and caecal junction) were cut, gently flushed with 0.9% saline solution, and placed in 10% neutral-buffered formalin. Additionally, intestinal digesta contents from the distal end of duodenum to ileo-caecal junction and pancreas samples were collected for protease activity measurement. The pancreas and intestinal content samples were collected from each bird within 5 min after death, placed in a 5-mL screw-capped tube and immediately frozen in liquid nitrogen and then stored at –80°C until further analysis.

Carcass traits

On d 36, two birds, based on the average body weight per pen, were randomly selected (eight birds/dietary treatment), leg-banded, and weighed individually. Birds were slaughtered by the Halal slaughtering process. When the birds were completely immobilized, carcasses were scalded at 55°C to 60°C

Table 1. Ingredient and nutrient compositions of experimental diets

Item	Starter (d 1-2)				Finisher (d 22-35)			
	NP[1]	LP1	LP2	LP3	NP	LP1	LP2	LP3
Ingredient (%)								
Corn	59.33	61.33	63.31	65.35	61.38	63.01	64.50	65.97
Soybean meal	32.40	27.15	21.95	16.65	27.75	22.35	17.00	11.75
Canola meal	1.00	3.00	5.00	7.00	1.50	4.00	6.50	9.00
Palm kernel meal	1.00	2.00	3.00	4.00	1.50	2.50	3.50	4.50
Palm olein	2.55	2.56	2.60	2.65	4.80	4.90	5.06	5.17
Limestone	1.31	1.32	1.32	1.31	1.12	1.11	1.11	1.10
Sodium chloride	0.34	0.34	0.33	0.33	0.34	0.35	0.34	0.34
MDCP	1.57	1.56	1.57	1.58	1.28	1.26	1.29	1.29
L-lysine	0.13	0.24	0.34	0.45	0.01	0.11	0.22	0.31
DL-methionine	0.21	0.23	0.26	0.29	0.18	0.21	0.23	0.25
L-threonine	0.06	0.12	0.17	0.23	-	0.06	0.11	0.16
L-tryptophan	-	-	-	0.01	-	-	-	0.02
Vitamin and mineral premix[2]	0.15	0.15	0.15	0.15	0.14	0.14	0.14	0.14
Nutrient (calculated, % unless stated otherwise)								
Metabolisable energy (MJ/kg)	12.71	12.71	12.71	12.71	13.31	13.31	13.31	13.31
Crude protein	21.00	19.74	18.50	17.24	19.05	17.87	16.70	15.58
Total amino acids								
Lysine	1.28	1.28	1.28	1.28	1.04	1.04	1.05	1.04
Methionine	0.53	0.54	0.56	0.57	0.48	0.50	0.51	0.52
Methionine+cysteine	0.89	0.89	0.89	0.89	0.82	0.82	0.82	0.82
Threonine	0.88	0.88	0.87	0.87	0.75	0.75	0.75	0.74
Tryptophan	0.26	0.24	0.22	0.21	0.24	0.22	0.20	0.20
Valine	1.03	0.96	0.90	0.83	0.95	0.88	0.82	0.76
Arginine	1.47	1.35	1.24	1.12	1.33	1.22	1.11	1.00
Phenylalanine+tyrosine	1.71	1.59	1.47	1.35	1.57	1.45	1.33	1.22
Leucine	1.81	1.70	1.59	1.47	1.68	1.57	1.46	1.35
Isoleucine	0.91	0.84	0.76	0.69	0.83	0.76	0.68	0.61
Histidine	0.57	0.53	0.49	0.45	0.52	0.48	0.45	0.41
Glycine+serine	1.89	1.75	1.62	1.47	1.72	1.59	1.46	1.33
Analysed composition (%)								
Crude protein	21.59	19.56	18.41	17.64	18.70	17.77	16.66	15.63
Lysine	1.28	1.15	1.14	1.17	1.09	0.96	1.04	0.97
Methionine	0.46	0.39	0.42	0.41	0.43	0.43	0.40	0.39
Threonine	0.85	0.84	0.82	0.86	0.78	0.75	0.78	0.72

MDCP, monodicalcium phosphate; AA, amino acid.
[1] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[2] Premix per kg of diet: vitamin A, 50 MIU: vitamin D_3, 10 MIU; vitamin E, 130 g; vitamin K_3, 10 g; vitamin B_1, 10 g; vitamin B_2, 25 g; vitamin B_6, 16 g; vitamin B_{12}, 0.1 g; biotin, 0.50 g; folic acid, 8 g; niacin, 200 g; pantothenic acid, 56 g; iron as ferrous sulphate monohydrate 110 g; zinc as zinc oxide 110 g; manganese as manganese oxide 110 g; copper as cupric sulphatepentahydrate 11 g; iodine as potassium iodate 2 g; cobalt as cobalt chelates of AA 0.3 g; selenium as selenium chelates of AA 0.3 g; chromium as chromium chelates of AA 0.5 g.

for 45 s and defeathered in a rotary plucker. The carcass weight was recorded with abdominal fat, lungs and kidneys but without giblets, feet or the head. Non-deboned breast meat with the attached skin (both pectoralis major and minor) and abdominal fat were removed and weighed. The abdominal fat pad was considered the tissue surrounding the gizzard and intestines, extending within the scheme, neighbouring the cloaca and bursa of fabricius, and adjoining the abdominal muscle.

Gut morphology

The intestinal segments (2 to 4 mm) were dehydrated for 16 h in an automatic tissue processor (Leica ASP 3000; Leica Biosystems GmbH, Nussloch, Germany) before being embedded in paraffin (Leica EG 1160; Leica Biosystems GmbH, Germany). Duplicate tissue sections with 4-μm thickness were produced using a rotary microtome machine (Leica RM 2155; Leica Biosystems GmbH, Germany). The sections were placed on a glass slide and heated at 57°C until dried. Upon drying,

the slides were stained with haematoxylin-eosin and covered with a cover-slip. Histological indices were analysed under a computer-aided light microscope using image analysis (Leica Camera AG, Solms, Germany). The VH (from the crypt mouth to the villus tip), CD (from the base up to the region of transition between the crypt and villi) and villus width (VW) (half of the villus length) were measured. Accordingly, the apparent ASA (VH×VW) and the VH/CD ratio were calculated [4].

Endogenous protease activity

Pancreas tissues were processed by adding five times their weight in ice-cold 0.2 M Tris buffer (pH 8.0) with 0.05 M NaCl and then homogenized using Ultra-Turrax homogenizer (IKA Works Inc., Wilmington, NC, USA). The homogenates were centrifuged at 3,000×*g* for 15 min at 4°C, and the supernatant was used for protease activity measurement as described by Jin et al [19]. Digesta samples were diluted 10 times (w/v) using ice-cold phosphate-buffered saline (pH 7.0) and then homogenized and sonicated for one min with three cycles at 30 s intervals. The samples were later centrifuged at 18,000×*g* for 20 min at 4°C, and the supernatants were used for the protease enzyme activity assay [19]. All procedures were handled on ice to prevent possible enzyme degradation. The protease activity unit was defined as milligrams of azocasein degraded during 2 h of incubation at 38°C per mg of intestinal digesta protein (U/mg protein) or pancreas. The intestinal digesta protein concentrations were determined using colorimetric reaction of the bicinchoninic acid protein assay kit (Sigma-Aldrich, St. louis, MO, USA).

Biochemical analysis

Feed samples were collected and finely ground using a coffee grinder (Panasonic, Shah Alam, Selangor, Malaysia). The dry matter (DM) and CP were determined following the AOAC methods [20], respectively. The AA concentrations were determined using pre-column derivatization with ACCQ reagent (6-aminoquinolyl-N-hydroxysuc-cinimidyl carbamate, Waters, Milford, MA, USA) and high-performance liquid chromatography [5]. Cysteine and methionine were analysed as cysteic acid and methionine sulfone by oxidation with performic acid for 16 h at 0°C. Serum concentrations of total protein (TP), albumin (ALB), uric acid (UA), and triglycerides (TG) were analysed by an automated chemistry analyser (Hitachi 902 Automatic Analyser; Hitachi, Tokyo, Japan) using commercial test kits (Roche Diagnostics, Basel, Switzerland).

Statistical analysis

The effects of CP level, enzyme supplementation and their interactions with growth performance, serum biochemical profile, carcass traits, intestinal morphology, and pancreatic and intestinal protease activity were analysed using the general linear models procedure in SAS (SAS Institute Inc., Cary, NC, USA). Duncan's multiple range test was used to compare between means. When interactions were significant, comparisons were made within each experimental variable. The effect of decreasing protein level was portioned into linear and quadratic components using orthogonal polynomial contrasts. Mortality data were subjected to a chi-square test. The significance is considered when $p<0.05$.

RESULTS

Growth performance

The effects of CP level and protease supplementation on FI, weight gain (WG), and FCR are presented in Table 2. There were no significant ($p>0.05$) CP×protease interactions for FI, WG, or FCR except FI for d 29 to 35. Except during the first week of life, FI was not affected by CP level during week 2 ($p = 0.07$) and 3 ($p = 0.523$). The FI was reduced linearly during d 1 to 7 ($p<0.001$) and d 22 to 28 ($p<0.001$) and d 29 to 35 ($p = 0.020$). The weekly data also showed that WG decreased linearly and FCR increased linearly by reduction of dietary CP. However, the results of WG and FCR were not significant for d 29 to 35. The FI and WG decreased linearly ($p<0.001$; $p<0.001$) and FCR increased linearly ($p<0.001$) with the reduction in dietary CP levels during d 1 to 35. The effect of protease supplementation was negligible on WG and FCR during the first 2 weeks of life. But thereafter the protease supplementation improved WG and FCR. Irrespective of dietary protein level, protease supplementation improved WG and FCR during d 1 to 21 ($p = 0.013$; $p = 0.002$) and d 1 to 35 ($p<0.001$; $p = 0.001$). The FI was not affected ($p>0.05$) by supplemental protease. Neither CP level nor enzyme supplementation had a significant effect on mortality rate (Table 2).

Serum biochemical profile

The effect of dietary CP level and protease supplementation on serum biochemicals is shown in Table 3. CP×protease interactions were significant for serum levels of ALB, TG, and UA but not for serum TP. Irrespective of protease supplementation, reducing the CP level linearly decreased ($p<0.001$) serum TP. Protease supplementation did not affect serum TP ($p = 0.749$). Serum ALB was linearly decreased by the dietary CP level reduction in both diets with and without protease ($p< 0.001$, $p = 0.042$, respectively) but was otherwise noted for TG and UA (Table 4). The level of TG increased linearly ($p<0.001$) with the reduction of dietary CP in the protease supplemented group. Within the non-protease-supplemented group, the level of UA decreased linearly ($p<0.001$) and quadratically ($p<0.07$).

Carcass traits

The CP level×protease interactions for carcass traits were not significant (Table 5). Reducing dietary CP linearly decreased the dressed yield ($p<0.001$) and breast meat ($p = 0.049$) and

Table 2. Effect of crude protein level and protease enzyme supplementation on growth performance in broiler chickens from day 1-35[1)]

Items	CP					Contrast, p-value		Protease			Probability		
	NP[2)]	LP1	LP2	LP3	SEM	Linear	Quadratic	With	Without	SEM	CP	Protease	CP×protease
Feed intake													
d 1-7	141	141	124	125	2.43	<0.001	0.966	131	135	1.62	<0.001	0.141	0.743
d 8-14	413	428	411	398	4.34	0.070	0.074	413	412	2.38	0.087	0.868	0.479
d 15-21	585	610	641	587	8.43	0.523	0.006	609	603	8.01	0.028	0.709	0.693
d 22-28	1,025	1,029	969	910	14.58	<0.001	0.084	977	990	10.03	<0.001	0.486	0.603
d 29-35	1,119	1,102	1,067	1,010	17.13	0.020	0.548	1067	1082	12.71	0.041	0.569	0.004
d 1-21	1,140	1,179	1,177	1,110	9.55	0.202	0.002	1152	1150	7.04	0.016	0.896	0.529
d1-35	3,284	3,309	3,214	3,029	35.57	<0.001	0.041	3196	3222	27.19	<0.001	0.564	0.058
Weight gain													
d 1-7	124	113	99	95	3.09	<0.001	0.084	106	109	1.22	<0.001	0.084	0.173
d 8-14	297	282	265	251	4.95	<0.001	0.963	277	270	3.31	<0.001	0.234	0.841
d 15-21	349	349	346	313	5.33	0.014	0.088	350 a	328b	7.03	0.023	0.025	0.870
d 22-28	505	484	487	456	10.39	0.125	0.793	508 a	459 b	14.39	0.380	0.021	0.986
d 29-35	619	609	522	491	15.96	<0.001	0.552	565	556	9.23	<0.001	0.617	0.174
d 1-21	770	743	711	658	11.35	<0.001	0.186	733 a	708 b	7.02	<0.001	0.013	0.984
d1-35	1,894	1,837	1,721	1,604	29.61	<0.001	0.207	1805 a	1723 b	18.60	<0.001	<0.001	0.188
FCR													
d 1-7	0.83	0.89	0.85	0.88	0.01	0.038	0.314	0.86	0.87	0.01	0.042	0.658	0.708
d 8-14	1.19	1.29	1.31	1.33	0.02	<0.001	0.115	1.27	1.29	0.01	<0.001	0.470	0.466
d 15-21	1.40	1.49	1.55	1.58	0.02	<0.001	0.034	1.48 b	1.53 a	0.01	<0.001	0.002	0.191
d 22-28	1.64	1.74	1.73	1.75	0.02	0.091	0.367	1.66 b	1.77 a	0.03	0.175	0.005	0.835
d 29-35	1.69	1.76	1.82	1.84	0.02	0.002	0.525	1.73 b	1.83 a	0.02	0.002	<0.001	0.090
d 1-21	1.48	1.59	1.66	1.69	0.02	<0.001	0.207	1.57 b	1.62 a	0.01	<0.001	0.002	0.204
d1-35	1.73	1.80	1.87	1.89	0.02	0.002	0.510	1.77 b	1.87 a	0.02	0.001	0.001	0.090
Mortality													
d 1-35	2.50	3.33	1.67	0.83	0.91	-	-	2.08	2.08	0.93	-	-	-

CP, crude protein; SEM, standard error of mean; FCR, feed conversion ratio.
[1)] Data represent mean values of 4 pens.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[a,b] Means within a row-subgroup without common superscripts differ at $p < 0.05$.

conversely increased the abdominal fat (p<0.001), regardless of protease supplementation. Protease supplementation increased carcass yield (p = 0.042), irrespective of dietary CP level. However, percentages of breast meat and abdominal fat pad were unaffected by protease supplementation.

Gut morphology

The effects of dietary CP level and protease supplementation on the VH, CD, VH/CD ratio and ASA in the duodenum, jejunum and ileum are displayed in Table 6. No significant CP×protease interactions were noted for duodenal VH, CD, VH/CD, and ASA, jejunal CD, VH/CD ratio and ASA and ileal VH, CD and VH/CD. However, significant CP level× protease interactions were found for jejunal VH and ileal ASA (p = 0.036). Irrespective of CP level, protease supplementation significantly increased duodenal ASA (p<0.001), jejunal VH/CD ratio (p<0.001) and ASA (p<0.001) and ileal VH (p<0.001). However, protease supplementation reduced jejunal CD (p = 0.008). Regardless of protease supplementation, jejunal VH (p<0.001), VH/CD (p = 0.004) and ASA (p<0.001) and ileal CD (p<0.001) decreased quadratically. However, ileal VH/CD increased quadratically (p = 0.020) with the reduction in dietary CP. In contrast, reducing dietary CP had no effect on duodenum VH/CD and ASA (p>0.05). Jejunal VH decreased linearly (p<0.001) and quadratically (p<0.001) with protease in LP diets, but only a quadratic decrease was observed when protease was supplemented. Ileal ASA increased linearly (p = 0.001) when protease was added to LP diets, whereas a quadratic decrease (p = 0.004) was observed among those without supplemental protease (Table 7).

Enzyme activity

No significant CP level×protease interactions were observed in pancreatic or intestinal protease activity (Table 8). Protease supplementation increased (p<0.001) intestinal protease activity and decreased (p = 0.041) pancreatic protease activity.

Table 3. Effect of crude protein level and protease enzyme supplementation on serum biochemical profile in broiler chickens at 35 days of age[1)]

Item	Albumin (g/L)	Triglycerides (mmol/L)	Total protein (g/L)	Uric acid (μmol/L)
Diets[2)]				
NP	11.88	0.54	30.11	248
LP1	11.33	0.74	26.83	219
LP2	10.93	0.76	26.59	249
LP3	9.73	0.86	23.78	150
SEM	0.21	0.04	0.54	11.62
Contrast, p-values				
Linear	<0.001	0.011	<0.001	0.167
Quadratic	0.314	0.531	0.777	0.146
Protease				
With	10.97	0.77	26.69	242[a]
Without	10.96	0.68	26.96	201[b]
SEM	0.18	0.04	0.43	9.75
Probabilities				
CP	<0.001	0.017	<0.001	<0.001
Protease	0.992	0.141	0.749	0.009
CP × protease	0.048	<0.001	0.320	<0.001

SEM, standard error of mean; CP, crude protein.
[1)] Data represent mean values of 8 birds.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[a,b] Means within a column-subgroup without common superscripts differ at $p<0.05$.

Table 5. Effect of crude protein level and protease enzyme supplementation on carcass traits (as % of live weight) in broiler chickens at 36 days of age[1)]

Item	Eviscerated[2)] (%)	Breastmeat (%)	Abdominal fat[3)] (%)
Diets[4)]			
NP	71.47	32.95	3.05
LP1	70.67	32.08	3.19
LP2	70.51	31.48	3.80
LP3	69.33	31.71	3.81
SEM	0.22	0.24	0.09
Contrast, p-values			
Linear	<0.001	0.049	<0.001
Quadratic	0.653	0.263	0.651
Protease			
With	70.90[a]	32.36	3.39
Without	70.09[b]	31.75	3.53
SEM	0.21	0.28	0.08
Probabilities			
CP	0.003	0.158	<0.001
Protease	0.042	0.208	0.366
CP × protease	0.237	0.452	0.868

SEM, standard error of mean; CP, crude protein.
[1)] Data represent mean value of 8 birds.
[2)] Carcass with abdominal fat, lungs and kidneys, but without giblets, paws and head.
[3)] Tissue surrounding the gizzard and intestines, extending within the scheme, neighbouring the cloaca and bursa of fabricius, and adjoining the abdominal muscle.
[4)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[a,b] Means within a column-subgroup without common superscripts differ at $p<0.05$.

Neither pancreatic nor intestinal protease activity was affected by dietary CP level.

DISCUSSION

Hot and relatively humid environmental constraint can generate a state of physiological and behavioural stress that might affect the nutrient requirement in broiler chickens which evoke a negative effect on their productive performance, immunity and survivability [7]. Beneficial use of LP diets with supple-

Table 4. Albumin, triglycerides, and uric acid in broiler chickens at 35 days of age when interactions between crude protein level and protease enzyme supplementation were significant[1)]

Serum metabolites	Diets[2)]					Contrast, p-values	
	NP	LP1	LP2	LP3	SEM	Linear	Quadratic
Albumin (g/L)							
With protease	12.09	11.54	11.29	8.95	0.31	<0.001	0.060
Without protease	11.66	11.13	10.56	10.50	0.22	0.042	0.581
Triglycerides (mmol/L)							
With protease	0.42	0.68	0.86	1.23	0.07	<0.001	0.630
Without protease	0.68	0.81	0.66	0.59	0.04	0.278	0.242
Uric acid (umol/L)							
With protease	182	290	323	159	15.71	0.717	<0.001
Without protease	315	157	164	141	16.42	<0.001	0.007

SEM, standard error of mean.
[1)] Data representmean value of 8 birds.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).

Table 6. Effect of crude protein level and protease enzyme supplementation on small intestinal morphology in broiler chickens at 35 days of age[1)]

Item	Duodenum				Jejunum				Ileum			
	VH (mm)	CD (mm)	VH/CD ratio (μm)	ASA (mm)	VH (mm)	CD (mm)	VH/CD ratio (μm)	ASA (mm)	VH (mm)	CD (mm)	VH/CD ratio (μm)	ASA (mm)
Diets[2)]												
NP	1,501	189	8.05	0.383	1,045	176	6.09	0.228	634	134	4.88	0.127
LP1	1,466	186	7.99	0.375	1,012	169	6.14	0.245	652	138	4.84	0.163
LP2	1,411	197	7.25	0.392	914	179	5.33	0.179	606	119	5.17	0.134
LP3	1,559	182	8.67	0.408	797	167	5.05	0.149	597	110	5.51	0.124
SEM	26.65	3.05	0.19	0.011	20.81	3.83	0.17	0.008	11.13	3.48	0.10	0.004
Contrast, p-values												
Linear	0.854	0.416	0.412	0.946	0.055	0.427	0.930	0.676	0.636	0.993	0.813	0.005
Quadratic	0.976	0.721	0.860	0.443	<0.001	0.960	0.004	<0.001	0.055	<0.001	0.020	0.041
Protease												
With	1,532	188	8.25	0.432[a]	960	161[b]	6.16[a]	0.219[a]	663[a]	129	5.26	0.146[a]
Without	1,436	189	7.72	0.346[b]	923	184[a]	5.15[b]	0.182[b]	582[b]	121	4.93	0.128[b]
SEM	17.94	1.71	0.12	0.014	9.20	5.34	0.20	0.006	13.34	3.78	0.09	0.003
Probabilities												
CP	0.220	0.431	0.025	0.805	<0.001	0.730	0.016	<0.001	0.141	0.053	0.060	<0.001
Protease	0.062	0.977	0.099	<0.001	0.090	0.008	<0.001	<0.001	<0.001	0.232	0.086	0.008
CP × protease	0.299	0.179	0.085	0.661	<0.001	0.532	0.621	0.173	0.258	0.555	0.093	0.036

VH, villus height; CD, crypt depth; ASA, absorptive surface area; SEM, standard error of mean; CP, crude protein.
[1)] Data represent mean value of 8 birds.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[a,b] Means within a column-subgroup without common superscripts differ at $p<0.05$.

mental EAA in a hot-weather has been reported [5,6]. In the present study, despite meeting the requirement for lysine, methionine+cysteine, tryptophan and threonine in LP diets, WG and FCR were depressed throughout the experimental period, regardless of protease supplementation. Reduction of growth performance in broilers fed LP diets and supplemented with EAA has been reported [5] irrespective of their environmental condition, and this reduction could be associated with lower levels of specific NEAA such as glycine and serine or glutamic acid, or with anon-specific need for nitrogen [6]. Earlier work on AA-supplemented LP diets in a hot environment [5] suggested that reduced FI could be a possible explanation for the inferior WG. The reduced FI was noted as a quadratic effect in this study, where only higher levels of CP reduction (LP2 and LP3) reduced FI markedly. This may possibly be due to one or several AA that are regulating FI other than CP concentrations per se [21]. Although protease supplementation improved WG and FCR, it failed to alter the linear reduction of WG and the increment of FCR incurred by reducing CP. This could be partly due to the lack of protease effect on FI. The protease used in this study was previously shown to enhance proteins, AAs and energy digestibility in broilers fed

Table 7. Intestinal morphology in broiler chickens at 35 days of age when interactions between crude protein level and protease enzyme supplementation were significant[1)]

Item	Diets[2)]				SEM	Contrast, p-values	
	NP	LP1	LP2	LP3		Linear	Quadratic
Jejunal villus height							
With protease	1,128	960	957	796	25.41	<0.001	<0.001
Without protease	962	1063	871	798	22.95	0.099	<0.001
Ileal absorptive surface area							
With protease	0.119	0.174	0.151	0.142	0.066	<0.001	1.000
Without protease	0.135	0.152	0.118	0.106	0.006	0.386	0.004

SEM, standard error of mean.
[1)] Data represent mean value of 8 birds.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).

Table 8. Effect of crude protein level and protease enzyme supplementation on endogenous protease activity in broiler chickens at 35 days of age[1)]

Item	Pancreas	Small intestinal
Diets[2)]		
NP	138.3	85.9
LP1	132.5	84.5
LP2	144.7	86.4
LP3	143.5	85.8
SEM	2.21	1.65
Contrast, p-values		
Linear	0.103	0.947
Quadratic	0.541	0.936
Protease		
With	136.0[b]	98.3[a]
Without	143.6[a]	73.0[b]
SEM	2.23	3.65
Probabilities		
CP	0.082	0.983
Protease	0.041	< 0.001
CP × protease	0.319	0.372

SEM, standard error of mean; CP, crude protein.
[1)] Data represent mean value of 8 birds.
[2)] NP, normal protein (starter CP21/finisher CP19); LP1, low protein diet 1 (starter CP19.7/finisher CP17.9); LP2, low protein diet 2 (starter CP18.5/finisher CP16.7); LP3, low protein diet 3 (starter CP17.2/finisher CP15.6).
[a,b] Means within a column-subgroup without common superscripts differ at $p < 0.05$.

different protein levels [12]. Therefore, the observed improvements in nutrient digestibility and nutrient retention [15,17,22] in birds fed diets containing exogenous protease may have beneficial effects on body weight. This finding is in accordance with those reported by Angel et al [11] and Mahmood et al [10], where supplementation of mono-component protease improved growth performance in broilers.

The data showed that descending levels of dietary CP reduced the concentrations of ALB and TP, irrespective of protease supplementation. Blood plasma TP and ALB are the main transport proteins in avian species, and they reflect the avian nutritional condition [23]. The reduction in serum TP and ALB could be attributed to deficiency in AA intake by the birds, as measured by the lower FI [5]. The linear reduction of FI (day 1 to 35) in our study may have accounted for the decreased UA level with the reduction in dietary CP level. The UA is a by-product of protein catabolism and turnover in the body. Thus, the decrease in UA could be associated with a deficiency of ingested AA. Furthermore, UA reduction may also be related to glycine deficiency, the substrate of UA synthesis in the liver [2,3]. It is interesting to note that the linear reduction in UA with decreasing dietary CP level was only noted among those without protease supplementation. It appears that protease supplementation may have released glycine in the digestive system and therefore compensated the shortage of glycine in LP diets [12]. On the other hand, serum TG was not affected by reduced protein diets containing no protease. However, decreasing CP with supplemental protease increased serum TG. This finding is in contrast to the findings of Abudabos [24]. The author fed broiler chickens with a commercial enzyme cocktail (protease and carbohydrase) and observed that enzyme supplementation has no effect on serum TG. This discrepancy may be due to the different types of enzyme used.

As expected, carcass and breast meat yield decreased, and the rate of abdominal fat deposition was significantly higher in birds that were fed LP diets in this study. Most of the previous studies reported the same phenomenon in broilers fed with LP diets [3,24]. This is due to a higher calorie:protein ratio in LP diets. It appears that the excess available energy beyond that required for protein deposition is diverted to abdominal fat deposition. Interestingly, although protease supplementation did not change the rate of fat deposition, it improved the carcass yield. There are conflicting reports on the effect of protease supplementation on carcass yield. While Rehman et al [22] noticed no significant change in carcass yield or abdominal fat, Abudabos [24] reported an increase in carcass and breast meat yield. The improvement in carcass yield associated with protease supplementation could be due to enhanced utilization and deposition of protein [23].

The present results concur with earlier work that reducing dietary CP was detrimental to gastrointestinal tract morphometry [4]. The decrease in the intestinal VH, VH/CD, and ASA is associated with poor nutrient absorption and consequently depressed growth performance in broiler chickens [17]. The adverse effect of LP diets on gut morphometry could be attributed to a decrease in the concentrations of NEAA such as glycine, glutamine and proline, which actively support the epithelial layer and represent a major fraction of intestinal masons and digestive secretions [25]. In contrast, protease supplementation significantly increased duodenal and jejunal ASA and jejunal VH/CD ratios and ileal VH. The present findings are in agreement with Cowieson et al [15], Ding et al [9] and Xu et al [17]. These results indicated that more nutrients were digested by supplemental protease [9,11], and most likely, the extra energy absorbed was used to improve the intestinal morphology.

It was noted that the reduction in dietary CP level did not alter pancreatic or intestinal protease activity in the present study. Ding et al [9], however, reported otherwise. On a cautionary note, Ding et al [9] reduced CP (21%, 20%, and 19% during starter and 19%, 18%, and 17% during grower phase) and supplemented with AA (lysine, threonine, and methionine). However, the calculated and analysed AA were lowered in LP diets. Thus, the discrepancy between pancreatic and intestinal protease activities may be due to insufficient supplementation of AA. The current results confirmed those of Mahagna et al [13], which showed that enzyme supplementation (protease and amylase at higher dosage, 1,000 ppm)

suppressed endogenous pancreatic protease activity. Exogenous proteases may augment endogenous peptidases by increasing protein digestibility and hydrolysing proteinaceous anti-nutritional factors. In contrast, Ding et al [9] fed broiler chickens with three levels of protease (0, 150, and 300 ppm) and observed that protease concentration has no effect on either pancreatic or small intestinal protease activity at d 21. However, protease (at 300 ppm) significantly increased pancreatic protease activity at d 42. These inconsistencies could be attributed to variations in the age of birds, the dosage, or the type of protease used [26].

CONCLUSION

The present findings demonstrated that protease supplementation at the rate used in this study may offset the detrimental effects caused by reducing dietary CP on growth performance, intestinal morphology and carcass yield in broiler chickens raised under a hot and humid tropical condition. The magnitude of compensation may depend on the type and dosage of protease supplemented.

CONFLICT OF INTEREST

We certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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