Effects of protease supplementation of low protein and/or energy diets on growth performance and blood parameters in broiler chickens under heat stress condition

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ABSTRACT

The effects of feeding low dietary crude protein (CP) and/or metabolisable energy (ME) with or without supplemental protease on growth performance, carcass characteristics and physiological responses in broiler chickens were investigated under cyclic heat stress condition. A total of 350 day-old male broiler chicks were fed with one of the following seven experimental diets: (1) recommended-CP and recommended-ME (RPE, served as control); (2) recommended-CP and low-ME (RPLE); (3) recommended-CP and low-ME with protease (RPLEP); (4) low-CP and recommended-ME (LPRE); (5) low-CP and recommended-ME with protease (LPREP); (6) low-CP and low-ME (LPE) and (7) low-CP and low-ME with protease (LPEP). From 22 to 42 d of age, half of the chickens from each dietary group were exposed to 34 ± 1°C for 7 h daily (heat stress), whereas the other half were raised at constant 23 ± 2°C (normal temperature). Supplementation of protease to RPLE, LPRE and LPE diets had no significant effects on feed intake (FI), weight gain (WG) or feed conversion ratio (FCR). Diet had no effect on serum glucose, total protein, certain acute phase proteins (APPs), corticosterone or breast yield. Regardless of protease supplementation, heat stressed birds had significantly lower FI, WG and breast yield, and higher FCR, APPs and corticosterone compared to birds raised in normal temperature. In conclusion, dietary supplementation of protease to low CP and/or ME diets showed negligible effects on growth performance, carcass characteristic and physiological responses in broiler chickens under heat stress condition. The inclusion of microbial protease in broiler diets could be considered by poultry industry as an effective nutritional tool for reducing ME or CP, in order to decrease abdominal fat deposition, improve feed efficiency and increase the profit margin.

HIGHLIGHTS

- Protease supplementation has no specific help for broilers under heat stress.
- Feeding low CP and/or ME diet is not stressful for broiler chickens.

Introduction

Heat stress (HS) is one of the most challenging environmental stressors. Heat-stressed birds are characterised by a number of behavioural, metabolic and physiological changes to maintain body homeostasis (Gonzalez-Esquerra and Leeson 2006; Mack et al. 2013). Physiologically, HS may elevate the production of heat shock protein 70 (HSP70), serum levels of corticosterone (CORT) and certain acute phase proteins (APPs) such as alpha-1 acid glycoprotein (AGP), ovotransferrin (OVT) and ceruloplasmin (CPN) (Najafi et al. 2015; Olubodun et al. 2015; Zulkifli et al. 2018). For birds under HS condition, dietary CP reduction was reported to be beneficial as protein metabolism was associated with higher heat increment than carbohydrate or lipid metabolisms (Musharaf and Latshaw 1999; Ojano-Dirain and Waldroup 2002). Diets reduced in crude protein (CP) or metabolisable energy (ME) have received a lot of attention in poultry nutrition to minimise the feed cost and environmental impact in the past few decades (Rahman et al. 2002; Zaman et al. 2008). However, reduction of CP and or ME beyond certain levels may exert a negative effect on feed intake (FI) and growth rate (Zaman et al. 2008; Dairo et al. 2010; Awad et al. 2015, 2017). Although...
broiler chickens may adapt to diet of low-CP or ME content by eating more feed in an attempt to meet their nutrient requirements (Payne 1967; Leeson et al. 1996), reduction below a certain level may not be compensated (Attia et al. 2011). Moreover, for birds under HS condition, feeding low-CP diets could be detrimental to their feed conversion ratio (FCR) (Zulkifli et al. 2018). Therefore, optimising broilers performance under HS condition is one of the major challenges encountered by poultry producers and nutritionists (Sandercock et al. 2001; de Souza et al. 2016). Protease supplementation may improve CP and energy digestibility in low-CP diets (Angel et al. 2011; Fru-Nji et al. 2011). Despite the increase in energy digestibility (≥100 kcal/kg) in previous studies (Freitas et al. 2011; Fru-Nji et al. 2011; Kalmendal and Tauson 2012), researchers have not treated energy metabolisability as affected by proteases supplementation in much detail. The enzyme supplementation has been reported to enhance ME digestibility in low-ME diets (Freitas et al. 2011; Kamel et al. 2015). Earlier studies from this laboratory have demonstrated that protease supplementation significantly increased ileal digestibility of energy, CP and amino acids (AA) (Law et al. 2015) and improved FCR, weight gain (WG), carcase yield and intestinal absorptive surface area in broilers raised in a hot and humid tropical environment (Law et al. 2018). Hence, protease supplementation with an appropriate reduction in dietary CP and ME may not only improve bird’s performance under HS condition and ensure the maximum utilisation of nutrients but also reduce the cost of production. Therefore, the objective of the present study was to investigate the effects of feeding low dietary CP and/or ME with or without supplemental protease on growth performance, carcase characteristics and physiological responses in broiler chickens raised under cyclic HS conditions. The hypothesis was that protease supplementation would increase the digestibility of energy, crude protein and amino acids in diets that reduced in CP and/or ME, and that increases in nutrients digestibility would lead to the same growth performance like standard CP and ME diets. We also hypothesised that feeding proteases supplemented low CP and/or ME diets would mitigate the effects of heat stress, resulting in lower HSP70 expression, and CORT and APPs concentrations.

Materials and methods

Enzyme compositions and activity

The protease used in this experiment was a purified microbial protease (Cibenza DP100; Novus International Inc., St. Charles, MO). The enzyme is an alkaline serine endopeptidase protease derived from Bacillus licheniformis with a protease activity of 600,000 units/g. The recommended inclusion rate by the manufacturer is 300 units/g of feed. The protease activity was determined using the method described by Jin et al. (2000).

Birds and management

This study was undertaken following the guidelines of the Research Policy on Animal Ethics of the Universiti Putra Malaysia. A total of 350 one-day-old Cobb male broiler chicks were purchased from a local commercial hatchery. The chicks wing banded, weighed on arrival and randomly allocated in groups of 5 into 70 cages in a 3-tiered battery cages (60 × 60 × 45 cm, length × width × height) with a wire mesh floor. The cages were located inside two completely identical temperature-controlled chambers (9.1 × 3.8 × 2.3 m, length × width × height). The floor space was 0.1 m² per bird. Ambient temperature on day 1 was set at 32 ± 1°C and gradually decreased until 23 ± 1°C was reached by day 21. The average relative humidity during the experimental period ranged between 61 and 87%.

Experimental design and diets

The experimental design was a 2 × 7 factorial arrangement with two levels of temperature (normal and heat stress) and 7 experimental diets. From d 1, 350 birds (5 cages as 5 replicates) were allocated to one of the seven experimental diets as follow: (1) recommended-CP and recommended-ME (RPE, served as control diet); (2) recommended-CP and low-ME (PRLE); (3) recommended-CP and low-ME with protease (RPLEP); (4) low-CP and recommended-ME (LPRE); (5) low-CP and low-ME with protease (LPREP); (6) low-CP and low-ME (LPE) and (7) low-CP and low-ME with protease (LPEP) (Figure 1). Low-CP diets were formulated to be nutritionally marginal in dietary protein with the addition of commercially available feed-grade AA to ensure that total lysine (Lys), methionine (Met), threonine (Thr), valine (Val) and tryptophan (Trp) levels met the Cobb 500 nutrient recommendations (Tables 1 and 2). Birds had free access to feed (mash form) and water and kept under continuous lighting throughout the experiment. The treatments groups were replicated in both chambers.
Figure 1. The experimental design and experimental diets. RPE: recommended-CP and recommended-ME diet; RPLE: recommended-CP and low-ME diet; LPRE: low-CP and recommended-ME diet; LPE: low-CP and low-ME diet; Diets (except RPE) compounded was subsequently divided into two, one serving as the control and the other supplemented with the enzyme to serve as the test diet; Heat stress was started from d 22–42 in chamber B.

Table 1. Ingredient composition (as fed basis) of the starter and finisher diets.

| Ingredient          | Starter (d 1–21) | Finisher (d 22–42) |
|---------------------|------------------|-------------------|
|                     | Control | RPLE | LPRE | LPE | Control | RPLE | LPRE | LPE |
| Corn                | 55.25   | 56.52 | 63.66 | 64.93 | 55.67   | 56.90 | 63.40 | 64.69 |
| Soybean meal        | 31.20   | 30.95 | 23.62 | 23.39 | 23.47   | 23.26 | 16.50 | 16.23 |
| Canola meal         | 4.00    | 4.00  | 4.00  | 4.00  | 4.00    | 4.00  | 4.00  | 4.00  |
| Palm kernel meal    | 1.50    | 1.50  | 1.50  | 1.50  | 1.50    | 1.50  | 1.50  | 1.50  |
| Palm olein          | 4.34    | 3.31  | 3.05  | 2.02  | 6.91    | 5.89  | 5.72  | 4.70  |
| Limestone           | 1.29    | 1.29  | 1.34  | 1.33  | 1.06    | 1.08  | 1.10  | 1.10  |
| Sodium chloride     | 0.34    | 0.34  | 0.34  | 0.34  | 0.33    | 0.33  | 0.33  | 0.33  |
| MDCP                | 1.54    | 1.54  | 1.57  | 1.57  | 1.22    | 1.21  | 1.25  | 1.25  |
| L-lysine HCl        | 0.17    | 0.17  | 0.35  | 0.35  | 0.06    | 0.06  | 0.23  | 0.23  |
| DL-methionine       | 0.15    | 0.15  | 0.18  | 0.18  | 0.11    | 0.10  | 0.14  | 0.14  |
| L-threonine         | 0.07    | 0.08  | 0.18  | 0.18  | 0.02    | 0.02  | 0.13  | 0.13  |
| L-tryptophan        | 0.00    | 0.00  | 0.05  | 0.05  | 0.00    | 0.00  | 0.04  | 0.04  |
| L-valine            | 0.00    | 0.01  | 0.00  | 0.01  | 0.00    | 0.00  | 0.01  | 0.01  |
| Vitamin premix      | 0.03    | 0.03  | 0.03  | 0.03  | 0.03    | 0.03  | 0.03  | 0.03  |
| Mineral premix      | 0.10    | 0.10  | 0.10  | 0.10  | 0.10    | 0.10  | 0.10  | 0.10  |
| Antioxidant         | 0.02    | 0.02  | 0.02  | 0.02  | 0.02    | 0.02  | 0.02  | 0.02  |

Control: recommended-CP and recommended-ME diet; RPLE: recommended-CP and low-ME diet; LPRE: low-CP and recommended-ME diet; LPE: low-CP and low-ME diet; Diets (except RPE) compounded was subsequently divided into two, one serving as the control and the other supplemented with the enzyme to serve as the test diet; ME: Metabolisable energy; Antioxidant: Butyrated hydroxytoluene.

Heat treatment

From day 22–42, birds in one chamber were exposed to constant 23 ± 2°C (normal temperature), while the birds in the other chamber were exposed to 34 ± 1°C for 7 h daily (10:00–17:00) (heat stress). The temperature increment to 34°C took ~45 ± 5 minutes and that was the point recorded as the start of the heat treatment.

Measurements

Body weight and FI (cage basis) data were recorded in day 1, 21 and 42. Feed conversion ratio was calculated accordingly after adjustment on mortality. Mortality was recorded upon occurrence daily. On day 42, two birds per cage were randomly selected (10 birds per diet-temperature subgroup) and removed with minimum disturbance to cage mates and killed by neck cut according to the halal method (Farouk et al. 2014) and exsanguination blood samples were collected. Following the blood sampling, brain samples were collected and placed in 5 mL screw-capped tube and snap frozen in liquid nitrogen for quantification of HSP 70 density expression (Soleimani et al. 2012). The blood samples were centrifuged at 3000 × g at 4°C for 15 min. The obtained serum samples were stored at ~80°C until further analysis for glucose (GLU), triglycerides (TG), creatine kinase (CK), OVT, AGP, CPN and CORT. On day 43, two birds from each cage were selected (10 birds/diet-temperature subgroup) and weighed individually. Birds were slaughtered and defeathered in a rotary plucker. Non-deboned breast meat with the attached skin (both pectoralis major and minor) and abdominal fat were removed and weighed.

Determination of crude protein and amino acids

The CP content of the diets was determined following the procedure of AOAC (1990). The AA content was determined using high-performance liquid chromatography, as described in details by Awad et al. (2014).

Blood biochemical

The serum concentrations of blood biochemical were analysed with an automated chemistry analyser (Hitachi 902 Automatic Analyser; Hitachi, Tokyo, Japan) using commercial test kits (Roche Diagnostics, Basel, Switzerland): TP (Cat. No.: 11553836 316), TG (Cat. No.: 1148872 216), GLU (Cat. No.: 11447513 216) and CK (Cat. No.: 12132524 216).

Physiological stress indicators

The concentrations of CPN was measured using method for determining the rate of formation of a coloured product from CPN and the substrate, 1,4-phenylenediamine dihydrochloride and OVT using a radial immune diffusion method as previously
described in details (Zulkifli et al. 2014). The AGP concentration was determined using a commercial ELISA kit specific to chicken (Life Diagnostics Inc., West Chester, PA). The CORT was measured by a commercial high sensitivity EIA kit (AC-15F1, IDS, Boldon, UK) according to the manufacturer’s instructions. The level of HSP 70 expression was determined as previously described (Soleimani et al. 2012) with some modifications. Briefly, brain sample (0.3 g, whole cerebrum) was homogenised using a homogeniser (IKA Ultra-Turrax, Staufen, Germany) with 1.5 mL of protein extraction buffer (20 Mm Tris, pH 7.5; 0.75 M sodium chloride) and 10 μL/mL protease inhibitor cocktail (P8340, Sigma Chemical Co., St. Louis, MO) followed by centrifugation at 20,000 × g for 30 min at 4°C. The protein concentration and HSP 70 of the supernatants were quantified by the bicinchoninic acid protein assay kit (B9643, Sigma Chemical Co., St. Louis, MO). SDS-PAGE and Western Blotting were carried out and the final brain HSP 70 concentration was calculated as an arbitrary unit of band density relative to a total protein concentration of each sample.

Statistical analyses

All statistical analyses were carried out using the Statistical Analysis System Version 9.4 software (SAS Institute Inc., Cary, NC). One-way ANOVA was used to analyse the starter period (day 1–21) growth performance data. The growth performance data of the finisher (day 22–42) and overall (day 1–42) periods, APPs, CORT, HSP 70, serum metabolites and carcase traits data were subjected to two-way ANOVA using the General Linear Model (GLM) procedure of SAS to identify the main effects of diet, temperature and their interactions. When the interactions between the main effects were found to be significant, comparisons were made within each experimental variable. Comparison between means was done by Duncan’s multiple range test. Statistical significances are considered at p < 0.05.

Results

Growth performance and mortality rate

The analysed nutrient values of CP and AA in the starter and grower diets were in close agreement with the calculated values (Table 2). The analysed protease activity in the relevant experimental diets for the RPLEP, LPREP and LPEP diets were respectively 315, 296 and 283 in the starter diets and respectively 298, 324 and 310 in the finisher diets. The protease activity was not detected in the control, LPRE, RELE or LPE diets. Diet had no effect on FI (p = 0.572) or WG (p = 0.092) in the broilers during the starter period. However, the FCR of the birds fed on LPEP diet was significantly greater compared with the control, RPLEP and LPREP fed counterparts but no difference with those fed with the RPLE, LPRE and LPE diets (Table 3). Diet showed no significant effect (p > 0.05) on mortality rate from day 1 to 21 (Table 3). There were no significant diet × temperature interactions for the FI (p = 0.670; p = 0.750), WG (p = 0.592; p = 0.794) and FCR (p = 0.078; p = 0.452) in the broilers during the finisher and overall periods (Table 4). Diets

| Table 2. Nutrient composition of the starter and finisher diets. |
|------------------|-------------------------------|-------------------------------|-------------------------------|-------------------------------|
|                 | Control RPLE LPRE LPE        | Control RPLE LPRE LPE        |                 |-------------------------------|-------------------------------|-------------------------------|
| Item             | Nutrient (calculated, % unless stated otherwise) | Nutrient (calculated, % unless stated otherwise) |                 | Nutrient (calculated, % unless stated otherwise) | Nutrient (calculated, % unless stated otherwise) |                 |
|                  | ME, Kcal/kg 3035 2985 3035 2985 3180 3130 3180 3130 | Crude protein 21.00 21.00 18.50 18.50 19.00 19.00 16.70 16.70 |                 | Lysine 1.31 1.31 1.31 1.31 1.07 1.07 1.07 1.07 | Methionine 0.47 0.47 0.47 0.47 0.46 0.46 0.46 0.46 |                 |
|                  | Cystine 0.48 0.48 0.48 0.48 0.42 0.42 0.42 0.42 | Cystine 0.48 0.48 0.48 0.48 0.42 0.42 0.42 0.42 |                 | Tryptophan 0.27 0.27 0.27 0.27 0.24 0.24 0.24 0.24 | Threonine 0.88 0.88 0.88 0.88 0.76 0.76 0.76 0.76 |                 |
|                  | Valine 1.02 1.02 1.02 1.02 0.94 0.94 0.94 0.94 | Valine 1.02 1.02 1.02 1.02 0.94 0.94 0.94 0.94 |                 | Arginine 1.47 1.47 1.47 1.47 1.32 1.32 1.10 1.10 | Arginine 1.47 1.47 1.47 1.47 1.32 1.32 1.10 1.10 |                 |
|                  | Leucine 1.79 1.80 1.81 1.81 1.63 1.63 1.46 1.46 | Leucine 1.81 1.81 1.81 1.81 1.63 1.63 1.46 1.46 |                 | Isoleucine 0.91 0.91 0.77 0.77 0.81 0.81 0.68 0.68 | Isoleucine 0.91 0.91 0.77 0.77 0.81 0.81 0.68 0.68 |                 |
|                  | Histidine 1.30 1.29 1.05 1.05 1.07 1.07 0.83 0.83 | Histidine 1.05 1.05 1.05 1.05 1.07 1.07 0.83 0.83 |                 | Glycine + Serine 1.91 1.91 1.63 1.63 1.72 1.72 1.47 1.47 | Glycine + Serine 1.91 1.91 1.63 1.63 1.72 1.72 1.47 1.47 |                 |

Analysed composition, %

| Item            | Control RPLE LPRE LPE | Control RPLE LPRE LPE |                 | Control RPLE LPRE LPE |
|-----------------|-----------------------|-----------------------|-----------------|-----------------------|
| Crude protein   | 21.38 21.22 17.64 17.82 | 18.27 18.58 16.67 17.03 |                 |                      |
| Lysine          | 1.31 1.29 1.38 1.08 | 0.99 1.01 0.98 0.98 |                 |                      |
| Methionine      | 0.50 0.47 0.50 0.44 | 0.46 0.41 0.40 0.44 |                 |                      |
| Threonine       | 0.95 0.96 1.01 0.84 | 0.75 0.79 0.78 0.83 |                 |                      |

Control: recommended-CP and recommended-ME diet; RPLE: recommended-CP and low-ME diet; LPRE: low-CP and recommended-ME diet; LPE: low-CP and low-ME diet; Diets (except RPE) compounded was subsequently divided into two, one serving as the control and the other supplemented with the enzyme to serve as the test diet.

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had no effect on the finisher FI ($p = .446$), finisher WG ($p = .084$) or the overall FI ($p = .563$). Birds fed by LPE and LPEP diets had poorer overall WG ($p = .043$) compared with those fed the control and RPLEP diets. However, no significant differences were found among the groups fed on RPLE, LPRE and LPREP diets. The finisher FCR ($p = .003$) of the birds fed on LPE diet were significantly greater than their RPLE and RPLEP counterparts but not different from the groups fed with the control, LPRE and LPREP diets. A similar trend of change was observed in the birds fed on diets LPRE and LPREP. Heat challenge reduced the FI ($p < .001$; $p < .001$) and WG ($p < .001$; $p < .001$) but increased the FCR ($p = .001$; $p = .014$) in the finisher and overall periods when compared to unchallenged birds. Neither diet nor heat treatment had significant effect ($p > .05$) on the mortality rate during the finisher period (Table 4).

### Serum metabolites

There were no significant diet × temperature interactions for GLU ($p = .696$), TG ($p = .105$) or TP ($p = .355$). Diet had no effect on serum concentrations of GLU ($p = .173$) and TP ($p = .068$). Birds fed on the control,
Table 5. Effects of diet and temperature on serum metabolites in broiler chickens at 42 days of age.

| Item       | GLU, mmol/L | TG, mmol/L | TP, g/L | CK, U/L |
|------------|-------------|------------|---------|---------|
| Diet       |             |            |         |         |
| Control    | 14.740      | 0.460      | 26.660  | 4115    |
| RPLE       | 14.930      | 0.500      | 27.110  | 3714    |
| RPLEP      | 15.400      | 0.400      | 28.980  | 5211    |
| LPRE       | 15.400      | 0.690      | 27.490  | 5344    |
| LPREP      | 14.850      | 0.720      | 24.660  | 5244    |
| LPE        | 15.720      | 0.720      | 27.950  | 4955    |
| LPEP       | 15.930      | 0.700      | 27.690  | 5177    |
| SEM        | 1.640       | 0.250      | 3.960   | 1383    |
| Temperature|             |            |         |         |
| Normal     | 14.450      | 0.460      | 25.760  | 4498    |
| Heat stress| 16.040      | 0.740      | 28.740  | 5179    |
| SEM        | 1.450       | 0.240      | 3.800   | 1442    |

Analysis of variance (p value)

- Diet .173
- Temperature <.001
- Diet x temperature <.001

| Analysis of variance (p value) | <.001 | <.001 |
|-------------------------------|-------|-------|
| Diet                          |       |       |
| Temperature                   |       |       |
| Diet x temperature            |       |       |

Data represent mean values of 10 birds per treatment.

RPLE and RPLEP diets had lower (p < .001) TG as compared with those fed on LPRE, LPREP, LPE and LPEP diets. Regardless of diet, heat stressed birds had significantly higher GLU (p < .001), TG (p < .001) and TP (p < .001) levels than their counterparts maintained in normal ambient temperature. At 42 days of age, there were significant diet x temperature interactions for CK (p = .002). Diet had no significant effect on CK levels among the heat stressed chickens (Table 5). However, under normal temperature, birds fed with the control and RPLE diets had lower CK levels than other groups (Table 6). In addition, temperature produced no significant effect on CK levels among the control, LPRE, LPREP, LPE and LPEP fed birds.

Table 6. Creatine kinase where the interaction between diet and temperature were significant.

| Diet       | Normal | Heat stress | SEM  | p value |
|------------|--------|-------------|------|---------|
| Control    | 3502b  | 4881        | 1656 | .098    |
| RPLE       | 2430c  | 4869        | 1320 | .001    |
| RPLEP      | 4724d  | 5753*       | 984  | .036    |
| LPRE       | 5018e  | 5670        | 1026 | .172    |
| LPREP      | 5299f  | 5183        | 1297 | .849    |
| LPE        | 4803g  | 5107        | 1437 | .788    |
| LPEP       | 5612h  | 4618        | 1031 | .076    |
| SEM        | 1397i  | 1131        | 314  | .314    |

p < .001

There were no diet x temperature interactions for the percentage of breast yield and abdominal fat in broiler chickens at 43 days of age (Table 7). The breast meat yield (p = .136) was not affected by diet. Birds fed with RPLEP diet showed lesser (p < .001) abdominal fat when compared to other groups. Birds fed using LPRE, LPREP and LPE diets had greater abdominal fat compared to those fed control and RPLE. Regardless of diet, the abdominal fat was not affected (p = .328) by heat treatment. However, heat treatment had a significant effect on the breast meat yield as breast meat yield was lower in heat stressed birds compared to their counterparts kept in normal temperature.

Table 7. Effects of diet and temperature on the percentage of breast yield and abdominal fat in broiler chickens at 43 days of age.

| Item       | Breast yield, % | Abdominal fat, % |
|------------|-----------------|------------------|
| Diet       |                 |                  |
| Control    | 34.990          | 2.840            |
| RPLE       | 34.770          | 2.930            |
| RPLEP      | 35.690          | 3.640            |
| LPRE       | 34.670          | 3.560            |
| LPREP      | 34.430          | 3.500            |
| LPE        | 34.880          | 3.580            |
| LPEP       | 33.670          | 3.190            |
| SEM        | 2.210           | 0.670            |

Temperature

| Normal     | 35.300          | 3.210            |
| Heat stress| 34.160          | 3.090            |
| SEM        | 2.170           | 0.780            |

Analysis of variance (p value)

- Diet .136
- Temperature <.001
- Diet x temperature .213

Data represent mean values of 10 birds per treatment.

Physiological stress indicators

There were significant diet x temperature interactions for HSP70 (p = .002), but not for CPN (p = .100), OVT (p = .792), AGP (p = .254) or CORT (p = .884) in broilers at 42 days of age (Table 8). Diet showed no significant effect on HSP70 expression among birds raised in a normal temperature condition. However, under HS condition, birds fed control and RPLE diets had significantly lower HSP70 expression than those fed other diets (Table 9). Diet had no significant effect on CPN (p = .424), OVT (p = .814), AGP (p = .083) and CORT (p = .465). Irrespective of diet, serum levels of CPN, OVT, AGP and CORT were greater (p < .001) in heat
challenged birds compared to their unchallenged counterparts.

**Discussion**

The present findings suggested that protease supplementation had a negligible effect on the performance of broilers compared to those without enzyme supplementation. This outcome is on the contrary to those of Abudabos (2012) who found that enzyme supplementation was able to restore the nutritional value of diets with low CP and ME in broilers. These discrepancies could be attributed to the type of enzyme supplemented. Abudabos (2012) used a multi-enzyme supplement containing an acidic protease, α-amylase, pectinase, phytase, glucoamylase, cellulase and Aspergillus awamori cells, the present experiment used a mono-component protease. Data on the effect of mono-component protease on growth performance of broilers are limited and inconsistent. Several studies (Angel et al. 2011; Freitas et al. 2011; Cowieson et al. 2017; Mahmood et al. 2017a,b) have shown that supplementation of protease improved BW, FI and FCR in broilers. The improvement in growth performance was mainly attributed to the enhancement of CP and AA digestibility following protease supplementation. Protease used in the present study enhanced apparent ileal digestible energy (AIDE), CP and AA in a previous study (Law et al. 2015). However, the same improvements were not observed in this study. The present results concur with Ghazi et al. (2002) and Kaczmarek et al. (2014) who reported no improvement or poorer growth performance in broilers fed diets supplemented with protease. The authors attributed their observation to the possible negative effects of exogenous protease supplementation on secretion of the endogenous proteolytic enzymes. Consistent with this hypothesis, the protease used in the current experiment noted to decrease the endogenous pancreatic protease secretion in an earlier experiment (Law et al. 2018).

In the present experiment, a significantly lower WG and poorer FCR was observed when birds were given LPE and LPEP diets compared to those received the

| Table 8. Effects of diet and temperature on serum ceruloplasmin, ovotransferrin, α1-acid glycoprotein, corticosterone and brain heat shock protein70 in broiler chickens at 42 days of age. |
| --- |
| Item | CPN, mg/mL | OVT, mg/mL | AGP, mg/mL | CORT, ng/mL | HSP 70, arbitrary unit |
| **Diet** | | | | | |
| RPE | 0.790 | 0.260 | 2.360 | 1.930 | 0.560<sup>c</sup> |
| RPLE | 0.980 | 0.300 | 2.780 | 1.760 | 0.570<sup>bc</sup> |
| LPRE | 0.970 | 0.290 | 2.680 | 1.610 | 0.570<sup>bc</sup> |
| LPREP | 0.890 | 0.260 | 1.780 | 2.260 | 0.610<sup>bc</sup> |
| LPE | 0.730 | 0.270 | 1.700 | 2.330 | 0.600<sup>ab</sup> |
| LPEP | 0.940 | 0.280 | 1.970 | 2.280 | 0.610<sup>ab</sup> |
| SEM | 0.760 | 0.080 | 1.560 | 1.610 | 0.110 |
| **Temperature** | | | | | |
| Normal | 0.610<sup>b</sup> | 0.240<sup>b</sup> | 1.670<sup>b</sup> | 1.050<sup>b</sup> | 0.510<sup>b</sup> |
| Heat stress | 1.160<sup>a</sup> | 0.320<sup>a</sup> | 2.860<sup>a</sup> | 3.010<sup>a</sup> | 0.670<sup>a</sup> |
| SEM | 0.700 | 0.070 | 1.470 | 1.260 | 0.080 |
| Source of variation | | | | | |
| Diet | | | | | |
| Control: recommended-CP and recommended-ME; RPLE: recommended-CP and low-ME; LPRE: low-CP and recommended-ME; LPREP: low-CP and recommended-ME with protease; LPE: low-CP and low-ME; LPEP: low-CP and low-ME with protease; SEM: standard error of the mean; CPN: ceruloplasmin; OVT: ovotransferrin; AGP: α1-acid glycoprotein; CORT: corticosterone; HSP: heat shock protein.

| Table 9. Heat shock protein 70 where the interaction between diet and temperature were significant. |
| --- |
| Item | Heat shock protein 70 |
| **Normal** | | |
| Diet | 0.523<sup>y</sup> | 0.588<sup>x</sup> | 0.040 | .006 |
| RPLE | 0.330<sup>y</sup> | 0.603<sup>x</sup> | 0.070 | .037 |
| LPRE | 0.465<sup>y</sup> | 0.672<sup>**</sup> | 0.080 | <.001 |
| LPEP | 0.517<sup>y</sup> | 0.705<sup>**</sup> | 0.060 | <.001 |
| LPE | 0.536<sup>y</sup> | 0.722<sup>**</sup> | 0.090 | <.001 |
| LPEP | 0.511<sup>y</sup> | 0.702<sup>**</sup> | 0.070 | <.001 |
| SEM | 0.070 | 0.060 | | |
| p value | .355 | <.001 |
| **Heat stress** | | |
| Diet | 0.523<sup>y</sup> | 0.588<sup>x</sup> | 0.040 | .006 |
| RPLE | 0.330<sup>y</sup> | 0.603<sup>x</sup> | 0.070 | .037 |
| LPRE | 0.465<sup>y</sup> | 0.672<sup>**</sup> | 0.080 | <.001 |
| LPEP | 0.517<sup>y</sup> | 0.705<sup>**</sup> | 0.060 | <.001 |
| LPE | 0.536<sup>y</sup> | 0.722<sup>**</sup> | 0.090 | <.001 |
| LPEP | 0.511<sup>y</sup> | 0.702<sup>**</sup> | 0.070 | <.001 |
| SEM | 0.070 | 0.060 | | |
| p value | .355 | <.001 |

<sup>x,y</sup>Means within a column without common superscripts differ at p < .05. <sup>a,b</sup>Means within a row without common superscripts differ at p < .05. Control: recommended-CP and recommended-ME; RPLE: recommended-CP and low-ME; LPRE: low-CP and recommended-ME; LPREP: low-CP and recommended-ME with protease; LPE: low-CP and low-ME; LPEP: low-CP and low-ME with protease. Control: recommended-CP and recommended-ME; RPLE: recommended-CP and low-ME; LPRE: low-CP and recommended-ME; LPREP: low-CP and recommended-ME with protease; LPE: low-CP and low-ME; LPEP: low-CP and low-ME with protease.
control diet. These effects were more obvious during the overall period rather than the starter phase. Thus, it appears that nutrient density is more critical during the finisher rather than the starter period (Kamran et al. 2008; Cowieson et al. 2017). Interestingly, no reduction in FI and WG, and elevation in FCR were observed when either protein (LPRE and LPREP) or energy (RPLE and RPLEP) was reduced. These results are in agreement with previous findings that a reduction of dietary CP (in essential AA fortified diet) up to 3% (Bregendahl et al. 2002; Si et al. 2004; Namroud et al. 2008; Awad et al. 2014) or energy up to 100 kcal/kg ME (Zaman et al. 2008; Dairo et al. 2010) had no adverse effects on growth performance in broiler chickens.

As expected, irrespective of dietary treatment, high ambient temperature adversely affected growth performance of birds (Temim et al. 2000; Lin et al. 2006). However, feeding LPRE, LPREP, RPLE or RPLEP diet had no detrimental effects on the bird’s performance under both normal and heat stress conditions. Zaman et al. (2008) reported a beneficial effect of feeding low-CP at recommended ME diet under HS, outcomes which disagree with many other works when low-CP (Alleman and Leclercq 1997; Zulkifli et al. 2018) or low-CP and low-ME diets (Attia and Hassan 2017) were fed to broilers under the same conditions. These inconsistencies could be associated with differences in the levels of dietary CP, supplemented AA and/or the severity of heat challenge involved.

Diet showed no effect on serum GLU and TP in the present study. Swennen et al. (2007) and Hada et al. (2013) reported that carbohydrate metabolism in broiler chicken was not affected by CP and ME levels in the diets. This may be due to the strict regulation of carbohydrate metabolism in the same birds to maintain the blood GLU level (Hada et al. 2013). Corzo et al. (2005) and Hernández et al. (2012) fed broiler chickens with low-CP diets (3–4% in CP) and observed no change in serum TP. Corzo et al. (2009) and (Ahmadi et al. 2015) commented that TP will only be affected when diets ingested by the animals are deficient in AA. Thus, it appears that meeting the AA requirement could be more important than the CP per se. However, feeding low-CP diets, as demonstrated in the current experiment lead to elevation of liver lipogenesis and thus TG. Similarly, Swennen et al. (2006) and Dehghani-Tafti and Jahanian (2016) reported that irrespective of energy density, birds grown on low-CP diets had higher TG. It appears that the TG level is associated with calorie/protein and the excessive energy intake above the requirement level resulted in higher TG and therefore higher abdominal fat deposition (Rosebrough and Steele 1985; Sterling et al. 2002; Malheiro et al. 2003; Swennen et al. 2007).

Abdominal fat is an unfavourable trait in carcass quality and reduces its acceptability by the consumers. The increase in abdominal fat deposition is a major disadvantage of feeding low-CP diet (Sklan and Plavnik 2002). However, the percentage of breast meat yield was not affected by diet in the present experiment. Similar results have been reported previously by van Nguyen and Bunchasak (2005) and Infante-Rodríguez et al. (2016). On the other hand, the percentage of breast meat yield was decreased in the heat stressed birds. Such reduction can be attributed to lower ribosomal capacity under HS condition that leads to a decreased rate of protein synthesis and deposition (Temim et al. 1998). Similar results have been reported by Geraert et al. (1996) and Zhang et al. (2012).

It is interesting to note that birds received low-CP diet had higher CK level than those of controls under normal temperature. Moreover, the heat challenge resulted in higher CK in the birds fed low-ME diets than those provided control diet. Serum level of CK is considered a myopathy (muscle breakdown) marker when there is cell membrane damage and permeability changes (Sandercock et al. 2001). In this study, unlike other diets, heat stress resulted in significantly higher levels of CK in birds fed RPLE and RPLEP diets. It is possible that the variations in dietary CP and energy among the dietary groups caused the higher CK level by increasing the muscle breakdown at the expense of muscle synthesis. The phenomenon could be attributed to the lower calorie/protein in the mentioned diets, and consequently lower availability of energy under such energy demanding stressful condition (Fagan et al. 1992; Gaine et al. 2006).

The current study demonstrated that HS elevated CORT and APPs (CPN, OVT and AGP) in broilers. It is well documented that heat exposure can elevate CORT (Mahmoud et al. 2004; Soleimani et al. 2011; Najafi et al. 2015), APPs (Najafi et al. 2015; Zulkifli et al. 2018) and HSP 70 expression (Mahmoud et al. 2004; Najafi et al. 2015; Zulkifli et al. 2018) in broilers. In the present study, reducing dietary CP and ME did not affect CORT level. Similarly, (Houshmand et al. 2012) showed that low-CP diet (reduction of 3% in dietary CP) did not influence CORT in broiler chickens under hot and humid environment. Little information is available on the effect of low-CP diet on APPs. Recently, Zulkifli et al. (2018) reported that AGP and OVT levels were reduced in broilers fed diets with
more than 3% reduction in CP. On the contrary, the present findings suggest that diet had a negligible effect on APPs. The inconsistent results could be attributed to the lower levels of dietary CP reduction (2.3%) in the current experiment. However, Awad (2016) indicated that 5% reduction in dietary CP did not affect the levels of APPs (CPN, OVT, and AGP) if the diet was supplemented with all essential AA and Glycine (Gly). Dietary CP and ME levels with and without protease supplementation had no effect on HSP70 expression under normal temperature. However, low-CP irrespective of ME and supplemental protease significantly elevated HSP 70 expression under the same condition. It is possible that the observed higher HSP70 in low-CP fed birds is attributed to the higher calorie/protein ratio in these diets. The higher calorie/protein may provide extra energy needed for the metabolic functions related to coping mechanisms such as HSP 70 synthesis to maintain the homeostasis and cell integrity (Mallouk et al. 1999).

Conclusions
The present findings indicated that, regardless of protease supplementation, dietary CP and ME can be reduced to 18.5% and 2985 kcal/kg, respectively with no adverse effect on the FI, WG and survivability rates in broilers. However, unlike the single reduction of dietary CP or ME, the combined reduction of CP and ME was detrimental to FCR in broilers during 1–42 days of age. Irrespective of dietary CP and ME, supplementation of protease had negligible influence on growth performance. Heat stress adversely affects the growth performance of broiler chickens, regardless of protease supplementation and dietary CP or ME. These findings suggest that protease supplementation is a potential nutritional strategy for the poultry industry to minimise the adverse effects of decreasing nutrient density and potentially reducing the feed cost without affecting the growth performance of broilers, particularly in reduced ME or CP diet.

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

Ethical Approval
The experimental protocol used in this study was conducted in full compliance with the Research Policy and Code of Practice for the Care and Use of Animal for Scientific Purposes of Universiti Putra Malaysia.

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