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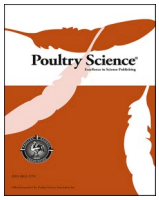

Full-Length Article

Multiprotease supplementation in laying hen diets: Impact on performance, egg quality, digestibility, gut histomorphology, and sustainability

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ABSTRACT

Laying hen responses to supplemental multiprotease on performance, egg quality, digestibility, gut histomorphology, nitrogen excretion, and economic performance of laying hens until 37 weeks of age were investigated. A total of 189 25-week-old Hy-Line Brown hens were housed in enriched cages (7 birds/cage) and randomly allocated to 1 of 3 diets with 9 replicates per treatment. Dietary treatments included: an adequate positive control- **PC** [met the breed and age standards for crude protein (**CP**) and amino acids (**AA**)]; negative control- **NC** (90 % CP and AA requirement); and NC supplemented with multiprotease – **NCMP**. Multiprotease was supplemented at 300 g/t of feed equating to 2400 U/kg. Egg production rate and feed intake were not altered ($P > 0.10$) by the dietary treatments. Between 25 and 37 weeks of age (**woa**), the NCMP diet reduced the feed conversion ratios by 3 % (1.91 vs 1.97; $P < 0.05$) in comparison to the NC diet while improving ($P < 0.05$) the egg weights by 3 % (58.56 vs 56.68); Haugh units by 2 % (91.78 vs 90.20); and breaking strength by 1 % (4.65 vs 4.61). Marginally intensified yolk color and albumen height ($P < 0.10$) were also observed with the NCMP diet. Furthermore, the NCMP diet marginally improved the villus height, width, and absorptive surface area ($P < 0.10$) relative to NC. Multiprotease-supplemented NCMP diet improved ($P < 0.05$) the digestibility of crude protein; and amino acids including lysine, methionine, phenylalanine, isoleucine, leucine, glutamine, tyrosine, relative to the NC diet. Lowered AA/CP diets (NC and NCMP) reduced the N excreted and feed costs ($P < 0.05$) relative to the PC diet. Multiprotease increased the returns on investment ($P < 0.10$), and nitrogen retained in egg ($P < 0.05$) from 25- 37woa. Conclusively, feeding reduced CP/AA diets maintained the egg production rate while reducing the N excreted and feed costs. Multiprotease modulation of ileal absorptive capacity and nutrient digestibility is linked to improved feed efficiency, egg quality, and revenue estimates of supplemented hens.

Introduction

Nutrient supply to meet requirements accounts for the largest share of poultry production costs, within which protein (i.e., AA) supply makes up the second largest portion. The feeding of reduced protein diets is targeted to lower feed costs and subsequently, maximize profits (Cai et al., 2024). Lowering dietary crude protein may also improve intestinal health through reduced hindgut fermentation of nitrogen fraction; along with reduced nitrogen excretion into the environment (Heo et al., 2013; Ji et al., 2014; Aderibigbe et al., 2024). Birds require specific quantities and balance of dispensable amino acids and even non-dispensable amino acids for efficient protein deposition. Consequently, the performance of birds fed reduced CP diets has been reported to be sub-optimal in broilers (Rehman et al., 2018; Barekatain et al., 2019); and layers (Dong et al., 2017; Cabezas-Garcia et al., 2022).

Protein digestion is primarily driven by the enzymatic hydrolysis of polypeptides by proteases. Although the avian gastrointestinal tract (**GIT**) has been described as short, simple, and highly efficient (Neves et al., 2014); and endogenous proteases being considered sufficient for optimal feed protein utilization, substantial evidence indicates the significant quantities of feed protein around 18-20 % that passes through the GIT without complete digestion (Ravindran et al., 2005;

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Ndazigaruye et al., 2019). This inherent inefficiency negatively affects avian health and contributes to environmental concerns through increased N excretion (Qaisrani et al., 2014; Oketch et al., 2023a). Exogenous protease supplementation is reasoned to optimize protein utilization more so, with poorly digestible feed ingredients, and is aimed at displacing expensive dietary protein sources in an attempt to maintain animal performance at reduced costs (Cowieson and Roos, 2016).

Exogenous protease is commonly supplemented in low CP/AA diets (Poudel et al., 2024); the rationale behind this approach is to provide enough room for protease-mediated improvements in the hydrolysis of dietary protein; and amino acid uptake; along with reduced nitrogen excretion into the environment. The expected improvements are targeted to restore the performance losses that could be brought about by low-protein diets. Supplemental use of proteases is well-reported in broilers (Liu et al., 2013; Duque-Ramírez et al., 2023; Penuela-Sierra et al., 2024). The potential of improved performance through multi-proteases with different optimal pH and substrate specificity relative to mono-component proteases has also been reported (Cho et al., 2020; Wealleans et al., 2023a). However, a lack of protease effect on performance has also been reported (Freitas et al., 2011; Walk et al., 2018) and the variabilities can be explained by differences in dietary factors including quality of the protein, type of feed ingredients, and composition of ingredients; and bird factors including age and genotype (Cowieson et al., 2016; Poudel et al., 2023).

Assessments for the specific efficacy of enzyme combination in dietary formulations with conventional and atypical feed ingredients across different poultry species are warranted to determine their specific efficacy. To the authors' knowledge, the efficacy of supplemental protease in laying hen nutrition has been scarcely explored. The current study investigates the effects of multiprotease supplementation on laying hen productive performance; internal egg and eggshell quality; dietary protein and amino acid digestibility; ileal histomorphology, nitrogen balance, and economic performance analyses of Hy-Line Brown layers during the early laying period. It was reasoned that multiprotease would maintain the laying performance; influence the interactions in the nutrient matrix to improve the internal egg and eggshell quality; and modulate the intestinal absorptive capacity for improved protein and amino acid digestibility, while reducing nitrogen excretion of laying hens fed reduced CP/AA diets. An additional hypothesis proposed that multiprotease supplementation would yield higher economic returns.

Materials and methods

The experiment was conducted at the Poultry Unit, Cheongyang Research Station of Chungnam National University. The experimental protocol and procedures were reviewed and approved by the Animal Ethics Committee of Chungnam National University (Protocol Number; 202307A-CNU-127).

Birds, and housing

A total of 189 23-week-old Hy-line Brown laying hens (1787.66 ± 9.34) from the same breeder flock were sourced from a commercial supplier (Ganongbio, Pocheon-si, Korea) and randomly allocated to 27 cages with 7 birds per cage. The hens were then provided with a two-week adaptation period and equally fed one of the three dietary treatments. During the adaptation phase, the laying rate of the birds was monitored and adjusted to ensure no significant differences between the groups before the formal start of the experiment. The hens were housed in enriched cages (90 cm long by 90 cm wide by 70 cm high) fitted with perches and nesting boxes as per welfare standards (European Commission, 1999). The birds were subjected to a lighting scheme with 16 h of continuous light and 8 h of darkness in a windowless and temperature-controlled (around 20-22°C) facility. Each cage was equipped with four nipple drinkers and a detachable feeder at the front for the provision of water and feed, respectively.

Experimental diets

The dietary treatments were formulated to be iso-energetic and included: positive control diet- PC (formulated to meet the breed and age standards for CP and AA); negative control diet- NC (formulated to achieve 90 % of the CP and AA requirement); and NC supplemented with multiprotease - NCMP, as shown in Table 1. The protease was mixed into the NC diet to create the NCMP diet. The protease tested is a commercial product (KEMZYME^{TM} Protease, Kemin Industries, Des Moines, IA) and was supplied at 2400 U/kg equating to 300 g/ton of feed. The inclusion levels were based on the supplier's recommendations. One unit of enzyme activity is defined as the amount of enzyme liberating 1 microgram of Azo-casein per minute at 37°C and pH 7.0 (Penuela-Sierra et al., 2024). The multiprotease combines acid (pepsin type protease), neutral (metallo- endopeptidase), and alkaline (serine endopeptidase), proteases, produced by *Aspergillus niger, Bacillus subtilis*, and *Bacillus licheniformis*, respectively.

Notably, the control diets (PC and NC) were mixed before the protease-supplemented NCMP diet to avoid cross-contamination. All the diets were in mash form. Feed bags were identified with the manufacturing date, net weight, bag number, and treatment code in the feed mill. Neither antibiotics, coccidiostats nor growth promotion additives were added to the diets. Precautions were taken during the collection and subsequent sample handling so that contamination did

Table 1
Ingredients and calculated nutrient composition of the basal diet.

Ingredients	Diets[1]	
	PC	NC
Corn	55.70	56.65
Wheat bran	2.02	5.80
Soybean meal, 44 %	26.90	21.47
Vegetable oil	1.65	1.62
Beef tallow	1.18	1.90
Limestone	9.57	9.57
Mono-calcium phosphate	1.85	1.85
Salt	0.50	0.50
DL-Methionine	0.21	0.19
Lys-HCl	0.12	0.15
Vitamin-mineral premix[2]	0.30	0.30
Calculated nutrient composition, %		
ME, kcal/kg	2850	2850
Crude protein	17.01	15.30
Crude fat	4.31	4.38
Linoleic acid	2.00	2.00
Crude fiber	3.37	3.48
Calcium	4.20	4.20
Total P	0.80	0.81
Available P	0.49	0.49
Linoleic acid	2.00	2.00
Calculated SID amino acid values		
Lysine	0.89	0.80
Methionine	0.44	0.40
Methionine + cysteine	0.74	0.66
Threonine	0.56	0.49
Tryptophan	0.17	0.15
Arginine	0.84	0.76
Isoleucine	0.61	0.53
Valine	0.70	0.62

[1] PC, adequate AA/CP; NC, 10 % reduced AA/CP.

[2] Provided per kilogram of diet: vitamin A (trans-retinyl acetate), 3,900,000 IU; vitamin D_3 (cholecalciferol), 1,500,000 IU; vitamin E (DL-α-Tocopherol acetate), 6200 IU; vitamin B1 (thiamin), 4.4 mg; vitamin K3 (menadione), 330 mg; vitamin B2 (riboflavin), 2600 mg; vitamin B5 (D-pantothenic acid), 2500 mg; vitamin B3 (nicotinic acid), 11,200 mg; vitamin B4 (choline), 140,000 mg; and vitamin B9 (folic acid), 275 mg; vitamin B6 (pyridoxine), 550 mg; vitamin B1 (thiamine), 440 mg; vitamin B7 (biotin), 12 mg; Fe (from iron sulfate), 90 mg; Cu (from copper sulfate), 8.8 mg; Zn (from zinc oxide), 40 mg; Mn (from manganese oxide), 54 mg; I (from potassium iodide), 0.35 mg; Se (from sodium selenite), 0.30 mg.

not occur. After feed mixing, composite samples from each batch and treatment were homogenized, labeled, and collected for evaluating the enzyme recovery analysis and proximate analysis.

Experimental procedures and sample collection

Animals and facilities were inspected twice daily for general health status, animal welfare, mortalities, constant water supply, temperature, lighting, humidity, ventilation, and unexpected events. All hens were individually weighed at 25 and 37 weeks of age to evaluate the impact of varying CP/AA levels and multiprotease supplementation on the body weight of the hens. Bi-weekly assessments for productive performance were conducted from week 27 to week 37. The total number and weight of eggs laid were recorded daily. The feed intake of the birds was adjusted according to the guidelines of the Hy-Line Brown Commercial Layers Management Guide (Hy-Line, 2024). Feed intake was recorded as the difference in the feed added throughout the week and the remaining feed in the feeder at the end of the week. Egg production was calculated based on hen-day egg production (**HDEP**), while egg loss percentages were determined by excluding eggs classified as spoiled or damaged, including those that were dirty, rough, misshapen, pimpled, cracked, white-banded, pale-shelled, soft-shelled, or shell-less (Nannoni et al., 2022). Additional performance metrics including the feed conversion ratio, and egg mass were also evaluated. At the end of weeks 29, 33, and 37, a total of 54 eggs (6 eggs per replicate cage) were collected randomly and evaluated for egg quality. Spoilt/damaged eggs were excluded during the egg quality evaluation. Internal egg quality analyses included the albumen height, Haugh units, yolk color, yolk percentage, and albumen percentage. The specific gravity, shell color, egg-breaking strength, shell thickness, and shell percentages were determined for the eggshell quality.

At the beginning of 37 weeks of age, the diets were mixed with 0.30 % chromic oxide as an indigestible marker and fed for about a week to evaluate nutrient digestibility. Subsequently, one bird per cage that was closer to the mean body weight was selected and euthanized by carbon dioxide asphyxiation (Ravindran et al., 2017). Abdominal incisions were made to separate the ileum and jejunum from the gastrointestinal tract. The jejunum was defined as the part before the vitelline diverticulum whereas the ileum was defined as the part of the ileum extending from the vitelline diverticulum towards the ileo-caecal junction. A cut was made of the ileum (30-40 mm) from the vitelline diverticulum and flushed with phosphate-buffered saline (**PBS**) at pH 7.4. The sample was then placed in labeled containers containing 10 % formaldehyde and stored for ileal histomorphological measurements including the villus height (**VH**), villus width (**VW**), crypt depth (**CD**), villus height to crypt depth ratio (**VH:CD ratio**) and absorptive villus surface area (**VSA**). After the separation and collection of the ileal samples, the digesta of the ileal segment from birds subjected to the same treatment was gently flushed with distilled water into labeled plastic containers, pooled by cage, and stored in a freezer at −20°C for dietary protein and amino acids digestibility analyses.

Regarding nitrogen excretion, excreta samples were collected at the end of weeks 29, 33, and 37 as per the procedure of Heo et al. (2023). All nine replicates and seven birds in a replicate cage were employed. Collected excreta samples were thoroughly mixed and pooled per replicate then stored at −20°C until further analyses including nitrogen intake; nitrogen excreted; and nitrogen retained as total, in egg or body. Economic performance parameters including the egg income; feed cost; and the returns on investment (**ROI**) were conducted at the end of weeks 31 and 37 and over the entire experimental period of weeks 25–37.

Sample processing and laboratory analyses

Excreta samples were oven-dried at 45°C for 96 h and finely ground for analyses. Feed samples were used for evaluating the enzyme recovery analysis (performed by Kemin Europa N.V., Toekomstlaan 42, 2200 Herentals, Belgium) and proximate analysis (performed by the Feed Analysis Center, Chungnam National University). Diets were analyzed for gross energy (bomb calorimeter), dry matter (930.15; AOAC, 2006), crude protein (990.03; AOAC, 2007), crude fat (920.39; AOAC, 2007), ash (942.05; AOAC, 2007) and amino acids (AOAC, 2006; method 982.30 E).

Eggshell breaking strength was evaluated using a texture analyzer (TA.XTplusC, Stable Micro Systems, Vienna Court, Lammas Rd, Godalming, Surrey, England). The eggshell color, albumen height, and Haugh units were measured using an egg multitester instrument (TSS QCM+ Range, Chessingham Park, Dunnington, York, England) featuring a digital balance, shell color reflectometer, albumen height gauge, internal Haugh Unit calculator, and a yolk colorimeter. The thick albumen height was read at least 1 cm from the yolk as recommended by Jones (2012). Yolk color intensity was measured against the DSM yolk color fan (1, light yellow; 15, orange).

Subsequently, the specific gravity of the eggs was determined by submerging the eggs in ascending order of a salty solution with a known specific gravity (Peebles and McDaniel, 2004). An egg yolk separator and weighing scale were then used for separating and weighing the yolk, albumen, and eggshell; their percentages relative to the total egg weight were subsequently determined. After internal egg quality analyses, the eggshells were collected, and any adhering albumen was removed using absorbent paper. The eggshells were then dried at room temperature for a day to determine the eggshell percentage relative to the egg weight. A shell thickness micrometer (Mitutoyo Digimatic MDC-MX Series, 965 Corporate Blvd, Aurora, Illinois 60502, USA) was then used to measure the shell thickness at three different locations (sharp, equator and blunt ends), with intact shell membranes. The internal egg quality and eggshell analyses were completed within 24 h of egg collection.

Regarding nutrient digestibility at the terminal ileum, the collected digesta samples were thawed and dried at 55°C for 24 h, ground, and strained through a 0.75-mm sieve (ZM 200 Ultra-Centrifugal Mill, Retsch GmbH & Co., KG, Haan, Germany) as detailed by Oketch et al. (2022). Nitrogen contents were determined using a combustion analyzer with EDTA as the calibration standard (model FP2000, Leco Corp., St. Joseph, MI). The digesta samples were analyzed for AA (AOAC, 2006; method 982.30 E). The chromium oxide concentration was determined as outlined by Fenton and Fenton (1979).

For the ileal histomorphology, ring-shaped lengths of the previously collected ileal samples that were fixed in 10 % formaldehyde were then excised, dehydrated, and embedded in paraffin wax. Then transverse sections (5μm) were cut, stained with hematoxylin-eosin then mounted on glass slides for viewing using an optical microscope (Olympus CX23, Olympus Corporation, Tokyo 163-0914, Japan). The villus heights, base widths, and crypt of Lieberkühn depths were measured through the analysis of the histological section images from the software (NIS-Elements Viewer software, Version: 4.20; NIS Elements, Nikon, USA). Cross-sections of 10 villi with prominent lamina propria were selected randomly for all the ileal morphology measurements. Villus height and width, as well as crypt depth, were measured at 100 × the objective magnification. All measurements taken from 10 villi per sample of an ileal segment were then expressed as the average for each hen. A total of 6 different transverse preparations per treatment were measured.

Calculations

Several parameters of productive performance including hen-day egg production, average daily feed intake, average egg weight, feed conversion ratio, egg mass, and egg loss percentage were calculated using the following equations (Oketch et al., 2024):

$$\text{Hen}-\text{day egg production, \%} = \frac{\text{Total number of eggs produced in a day}}{\text{Total number of laying hens in a day}} \times 100$$

$$\text{Average daily feed intake, g/hen/day} = \frac{\text{Total feed intake in a week}}{\text{Days} \times \text{Number of laying hens}}$$

$$\text{Average egg weight, g} = \frac{\text{Total egg weight}}{\text{Number of eggs}}$$

$$\text{Feed conversion ratio, g/hen/day} = \frac{\text{Feed intake per day per hen}}{\text{Average egg weight}}$$

$$\text{Egg mass, g/hen/day} = \frac{\text{Hen}-\text{day egg production} \times \text{egg weight}}{100}$$

$$\text{Egg loss percentage, \%} = \frac{\text{Number of spoilt eggs}}{\text{Number of total eggs}}$$

Haugh units were measured as follows:

$$\text{Haugh Units (HU)} = 100 \times \log(\text{AH} - 1.7\text{EW}^{0.37} + 7.6)$$

Where **AH** is albumen height in mm, and **EW** is egg weight in grams

The digestibility estimates for dietary crude protein and amino acids were calculated as follows (Oketch et al., 2022):

$$\text{Digestibility, \%} = 100 - \left[100 \times \left(\frac{\text{Marker conc.}_{\text{diet}} \times \text{Nutrient conc.}_{\text{digesta}}}{\text{Marker conc.}_{\text{digesta}} \times \text{Nutrient conc.}_{\text{diet}}}\right)\right]$$

where Marker conc.$_{\text{diet}}$ is the concentration of marker in the diet, and Nutrient conc.$_{\text{digesta}}$ is the nutrient concentration in ileal digesta whereas Marker conc.$_{\text{digesta}}$ is the marker concentration in ileal digesta, and Nutrient conc.$_{\text{diet}}$ is the nutrient concentration in the diet.

The villus height was defined as the distance from the villus tip to the junction of the villus and the crypt or simply the base of the lamina propria (Oketch et al., 2022). The midway point of the villus height was deemed to be representative of the average width between the apical and basal width (Oketch et al., 2022) and was used to obtain the villus width. Crypt depth was defined as the distance from the base of the villus to the muscularis mucosa. The ratio of the villus to crypt depth was estimated by dividing the villus height and mucosal crypt depth. Considering the villus as a cylindrical structure (Oketch et al., 2022), the villus surface area was calculated using the formulae:

$$\text{Villus absorptive surface area} = 2\pi \times \left(\frac{\text{Villus width}}{2}\right) \times \text{villus height}$$

Following the procedures outlined by Barzegar et al. (2019); De Cloet et al. (2023); and Miranda et al. (2015) for the nitrogen content of an egg at 1.936 (12.10 divided by 6.25), the nitrogen balance estimates for the nitrogen intake, excretion, and total nitrogen retained (**TNR**), nitrogen retention as egg (**NRegg**) or body (**NRbody**) were conducted as follows:-

$$\text{Nitrogen intake per day} = \text{Daily hen feed intake in grams} \times \left(\frac{\text{Diet N}}{100}\right)$$

$$\text{Total nitrogen retained} = \text{Nitrogen intake} - \text{nitrogen excretion}$$

$$\text{Nitrogen retained in egg} = 1.936 \times \text{egg mass}$$

$$\text{Nitrogen retained in body} = \text{total nitrogen retained} - \text{nitrogen retained in egg}$$

To estimate the egg income, the average egg weight was determined and categorized based on egg prices as of December 18th, 2023. The prices for Jumbo (above 68 g), extra-large (60 – 68 g), large (60 – 52 g), medium (52 – 44 g), and small size (less than 44 g) were estimated to average about $2.91, $2.79, $2.65, $2.52 and $1.98 per dozen eggs. The price per individual egg was then determined and adjusted to the hen-day egg production over two weeks. The feed income was determined as a factor of the feed ingredient and additive prices; and feed intake per hen. For the returns on investment, the difference between the egg income and the feed costs was determined.

Statistical analyses

Collected data were analyzed using the General Linear Model (**GLM**) procedure for the one-way ANOVA technique of IBM SPSS Statistics Windows, Version 26 (IBM Corp., Armonk, NY., USA) in a completely randomized design. The cage was used as the experimental unit for assessing the laying performance, egg quality, economic analysis, and nitrogen balance. Selected birds that were euthanized for sample collection were taken to be the experimental unit for the ileal nutrient digestibility and histomorphology. Least square means are reported, and orthogonal contrasts were used to assess linear responses to dietary treatments. Significance for the means was measured at $P < 0.05$ and marginal effects (tendency for significant effects) were measured at $0.05 < P < 0.10$. When significance was noticed for the treatment effects, means were separated using Tukey's Multiple Range Test.

Results

The analyzed dietary crude protein values for the NC and NCMP diets

Table 2
Analyzed chemical composition of experimental diets.

Items	Diets[1]		
	PC	NC	NCMP
Dry matter, %	88.78	89.10	88.68
GE, kcal/kg	3524	3568	3549
Crude protein, %	16.78	15.24	15.20
Crude fat, %	3.84	3.94	3.81
Crude fiber, %	3.42	3.48	3.48
Ash, %	6.45	6.56	6.71
Amino acids			
Lysine	1.05	0.99	0.89
Methionine	0.39	0.35	0.34
Methionine + cysteine	0.67	0.62	0.62
Threonine	0.70	0.66	0.68
Tryptophan	0.23	0.19	0.18
Arginine	1.12	1.07	1.04
Isoleucine	0.73	0.66	0.68
Valine	0.85	0.78	0.75
Histidine	0.45	0.42	0.40
Leucine	1.51	1.44	1.41
Phenylalanine	0.91	0.85	0.80
Alanine	0.89	0.86	0.86
Aspartic acid	1.78	1.66	1.51
Cysteine	0.28	0.27	0.27
Glutamine	3.11	3.03	2.98
Glycine	0.74	0.69	0.65
Proline	0.98	0.95	0.97
Serine	0.90	0.85	0.84
Tyrosine	0.56	0.48	0.49

[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.

Table 3
Effects of dietary CP/AA levels and protease supplementation on growth and productive performance of laying hens.

Items	Diets[1]			SEM[2]	P-Value[3]
	PC	NC	NCMP		
Growth performance					
Initial body weight, g/hen[4]	1786.19	1776.21	1800.57	9.340	0.568
Final body weight, g/hen[5]	1930.02	1927.55	1950.69	10.272	0.604
Body weight gain, g/hen[6]	148.83	151.33	150.12	14.482	0.975
Productive performance					
Week 25- 27					
Hen-day egg production %	90.98	90.47	92.51	3.298	0.968
Feed intake, g/d/hen	108.86	108.52	108.84	0.441	0.942
Feed conversion ratio, g feed/g egg	2.00	2.03	2.00	0.012	0.451
Egg weight, g	54.43	53.35	54.38	0.210	0.058
Egg mass, g/d/hen	49.47	48.27	50.29	1.787	0.901
Egg loss percentage, %	0.05	0.04	0.04	0.005	0.853
Week 27-29					
Hen-day egg production %	92.00	90.82	93.88	3.987	0.954
Feed intake, g/d/hen	109.33	109.99	110.51	0.283	0.238
Feed conversion ratio, g feed/g egg	1.86	1.92	1.90	0.014	0.215
Egg weight, g	58.96	57.34	58.19	0.402	0.267
Egg mass, g/d/hen	53.90	51.96	54.58	2.266	0.892
Egg loss percentage, %	0.05	0.05	0.04	0.005	0.833
Week 29-31					
Hen-day egg production %	93.87	93.53	96.94	3.100	0.890
Feed intake, g/d/hen	110.45	109.98	109.99	0.280	0.749
Feed conversion ratio, g feed/g egg	1.89[a]	1.94[b]	1.88[a]	0.009	0.032
Egg weight, g	58.58[b]	56.77[a]	58.47[b]	0.267	0.005
Egg mass, g/d/hen	54.99	53.09	56.51	1.797	0.748
Egg loss percentage, %	0.05	0.04	0.04	0.005	0.875
Week 31-33					
Hen-day egg production %	94.22	92.86	95.24	2.312	0.919
Feed intake, g/d/hen	111.68	111.63	111.78	0.307	0.981
Feed conversion ratio, g feed/g egg	1.87[a]	1.93[b]	1.91[ab]	0.009	0.018
Egg weight, g	59.73[b]	57.79[a]	58.70[ab]	0.229	0.001
Egg mass, g/d/hen	56.23	53.34	55.93	1.346	0.680
Egg loss percentage, %	0.04	0.04	0.04	0.007	0.932
Week 33-35					
Hen-day egg production %	94.05	90.99	94.90	3.431	0.892
Feed intake, g/d/hen	113.45	113.00	112.99	0.280	0.759
Feed conversion ratio, g feed/g egg	1.87[a]	1.98[b]	1.88[a]	0.018	0.021
Egg weight, g	60.74[b]	57.40[a]	60.40[ab]	0.560	0.023
Egg mass, g/d/hen	57.05	51.96	57.19	2.048	0.506
Egg loss percentage, %	0.03	0.05	0.05	0.005	0.690
Week 35-37					
Hen-day egg production %	93.88	90.31	95.58	2.464	0.683
Feed intake, g/d/hen	115.45	114.99	114.21	0.371	0.392
Feed conversion ratio, g feed/g egg	1.87[a]	1.98[b]	1.87[a]	0.020	0.001
Egg weight, g	61.53[b]	57.43[a]	61.20[b]	0.548	0.001
Egg mass, g/d/hen	57.59	51.43	58.44	1.383	0.065
Egg loss percentage, %	0.04	0.04	0.04	0.005	0.982
Week 25-37					
Hen-day egg production %	93.17	91.50	94.84	1.146	0.503
Feed intake, g/d/hen	111.53	111.35	111.39	0.207	0.932
Feed conversion ratio, g feed/g egg	1.89[a]	1.97[b]	1.91[a]	0.009	<0.001
Egg weight, g	58.99[b]	56.68[a]	58.56[b]	0.244	<0.001
Egg mass, g/d/hen	54.88	51.71	55.49	0.693	0.051
Egg loss percentage, %	0.04	0.04	0.04	0.005	0.986

[a-b]Means with different superscripts within the same column differ significantly.

[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.
[2] Pooled standard error of the mean.
[3] Statistical significance was determined at $P < 0.05$.
[4] Body weight as at 25 weeks of age.
[5] Body weight at 37 weeks of age.
[6] Calculated as the difference between the body weight of hens at 25 and 37 weeks of age.

corresponded to the formulated target of a 10 % reduction relative to the adequate PC diet (Table 2). The analyzed CP in the PC diet was 16.78 % whereas the values for the NC and NCMP diets were determined to be approximately 10 % lower at 15.24 %, and 15.20 %, respectively. Furthermore, the analyzed amino acid values corresponded to the formulated targets.

Table 4
Effects of dietary CP/AA levels and protease supplementation on egg quality of laying hens.

Items	Diets[1]			SEM[2]	P-Value[3]
	PC	NC	NCMP		
Week 29					
Egg-breaking strength, kg	4.49	4.55	4.56	0.156	0.942
Shell color	26.13	25.07	25.90	0.276	0.254
Egg specific gravity	1.10	1.07	1.09	0.009	0.514
Shell thickness, mm	0.36	0.36	0.36	0.133	0.911
Albumen height, mm	8.11	8.10	7.98	0.071	0.709
Haugh Units	92.06	92.47	91.87	0.227	0.542
Yolk color	7.77	7.80	7.93	0.100	0.402
Shell percentage, %	13.07	12.96	13.21	0.144	0.780
Yolk percentage, %	25.68	25.47	25.53	0.225	0.926
Albumen percentage, %	60.75	60.36	61.05	0.400	0.787
Week 33					
Egg-breaking strength, kg	4.49	4.59	4.56	0.132	0.857
Shell color	27.63	27.12	27.43	0.298	0.779
Egg specific gravity	1.10	1.09	1.10	0.017	0.970
Shell thickness, mm	0.36	0.36	0.36	0.103	0.973
Albumen height, mm	8.19	7.74	8.13	0.082	0.053
Haugh Units	92.26	90.40	92.05	0.315	0.029
Yolk color	7.61	7.57	7.67	0.119	0.071
Shell percentage, %	12.48	12.84	12.63	0.098	0.345
Yolk percentage, %	26.86	26.70	26.67	0.240	0.946
Albumen percentage, %	58.43	57.10	59.14	0.403	0.111
Week 37					
Egg-breaking strength, kg	4.35[a]	4.69[ab]	4.82[b]	0.164	0.023
Shell color	26.70	26.60	26.63	0.292	0.990
Egg specific gravity	1.09	1.10	1.11	0.017	0.956
Shell thickness, mm	0.37	0.36	0.35	0.088	0.359
Albumen height, mm	7.75	7.75	7.92	0.098	0.725
Haugh Units	90.31[ab]	87.73[a]	91.43[b]	0.504	0.008
Yolk color	8.32	8.27	8.40	0.099	0.790
Shell percentage, %	12.46	12.49	12.50	0.099	0.989
Yolk percentage, %	27.36	26.64	26.63	0.263	0.440
Albumen percentage, %	58.04	57.23	57.65	0.427	0.748
Week 25-37					
Egg-breaking strength, kg	4.44[a]	4.61[b]	4.65[b]	0.231	0.033
Shell color	26.82	26.26	26.66	0.176	0.412
Egg specific gravity	1.10	1.09	1.10	0.012	0.899
Shell thickness, mm	0.36	0.36	0.36	0.002	0.829
Albumen height, mm	8.02	7.87	8.01	0.051	0.398
Haugh Units	91.54[b]	90.20[a]	91.78[b]	0.216	0.005
Yolk color	7.90	7.87	8.00	0.067	0.064
Shell percentage, %	12.68	12.76	12.78	0.078	0.848
Yolk percentage, %	26.82	26.27	26.29	0.198	0.450
Albumen percentage, %	59.07	58.28	59.28	0.285	0.323

[a-b] Means with different superscripts within the same column differ significantly.

[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.
[2] Pooled standard error of the mean.
[3] Statistical significance was determined at $P < 0.05$.

Growth and productive performance

Multiprotease-supplemented NCMP diet reduced ($P < 0.05$) FCR at weeks 29-31, 31- 33, 33- 35, 35- 37; and the overall period from weeks 25- 37 relative to the NC diet (Table 3). Likewise, improved egg weights ($P < 0.05$) were observed with the protease-supplemented NCMP diet from weeks 29-31; 31- 33; 33- 35; 35- 37; and for the overall period from weeks 25- 37, relative to the NC diet. Marginal improvements ($P < 0.10$) in the egg weight from 25 to 27 weeks of age ($P = 0.058$); and egg mass from 35 to 37 weeks of age and the overall period from 25- 37 weeks of age ($P = 0.065$; $P = 0.051$, respectively) were also observed with the multiprotease- supplemented NCMP diet relative to the non-supplemented NC diet.

Internal egg and eggshell quality

Haugh Units were improved ($P < 0.05$); and albumen height and yolk color were improved ($P < 0.10$) with the multiprotease-supplemented NCMP diet relative to the non-supplemented NC diet at week 33 (Table 4). Furthermore, the multiprotease-supplemented NCMP diet improved ($P < 0.05$) the Haugh units and egg breaking-strength relative to the NC and PC diets at week 37. Multiprotease-supplemented NCMP improved ($P < 0.05$) the overall Haugh units and egg-breaking strength ($P < 0.05$); and marginally ($P < 0.10$) the yolk color from weeks 25 to 37.

Table 5
Effects of dietary CP/AA levels and protease on ileal histomorphology and nutrient digestibility of laying hens.

Items	Diets[1]			SEM[2]	P-Value[3]
	PC	NC	NCMP		
Ileal histomorphology					
Villus height (μm)	1002.96	833.57	994.07	33.241	0.054
Crypt depth (μm)	195.04	176.52	198.16	7.080	0.428
Villus width (μm)	103.68	89.41	122.09	6.284	0.098
Villus height: crypt depth (μm)	5.28	4.75	5.15	0.189	0.657
Villus absorptive surface area (mm^2)	0.33	0.24	0.38	0.027	0.081
Ileal nutrient digestibility					
Crude protein	82.33^{b}	73.40^{a}	81.76^{b}	1.139	0.010
Indispensable amino acids					
Arginine	81.99	74.91	80.17	1.193	0.071
Histidine	72.07	68.58	72.95	0.913	0.152
Isoleucine	73.17^{ab}	67.88^{a}	81.13^{b}	1.958	0.044
Leucine	78.37^{ab}	72.73^{a}	84.71^{b}	1.429	0.013
Lysine	73.93^{ab}	71.29^{a}	78.61^{b}	0.936	0.019
Methionine	79.83^{ab}	71.93^{a}	78.71^{b}	1.155	0.028
Phenylalanine	81.42^{b}	70.06^{a}	78.77^{ab}	1.774	0.048
Threonine	76.46	73.90	79.83	2.184	0.552
Tryptophan	80.12	73.08	80.01	1.377	0.088
Valine	79.27	73.48	80.97	1.824	0.246
Dispensable amino acids					
Alanine	77.98	72.99	80.74	1.680	0.195
Aspartic acid	78.98	75.23	84.55	2.000	0.194
Cysteine	76.64	73.17	77.88	2.134	0.654
Glutamine	84.53^{b}	74.18^{a}	80.22^{ab}	1.276	0.016
Glycine	82.44	76.12	82.38	2.455	0.499
Proline	74.38	72.90	76.09	1.178	0.555
Serine	80.93	71.92	83.78	2.287	0.121
Tyrosine	85.21^{b}	75.94^{a}	79.16^{ab}	1.195	0.020

[1)]Values are the mean of six replicates per treatment.
[a-b)] Means with different superscripts within the same column differ significantly.
[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.
[2] Pooled standard error of the mean.
[3] Statistical significance was determined at $P < 0.05$.

Ileal nutrient digestibility and histomorphology

The villus height, villus width, and absorptive surface area were improved ($P = 0.054$; $P = 0.098$; $P = 0.081$, respectively) with the multiprotease-supplemented NCMP diet relative to NC diet (Table 5). Regarding nutrient digestibility, the multiprotease-supplemented NCMP diet improved ($P < 0.05$) the digestibility of dietary crude protein; and several amino acids including lysine, methionine, phenylalanine, isoleucine, leucine, glutamine, tyrosine, when compared to the NC diet (Table 5). Tendency for multiprotease-mediated improvements ($P < 0.10$) was also noticed for the digestibility of arginine and tryptophan with the NCMP diet relative to the NC diet.

Nitrogen balance

Lowering the dietary AA/CP in the NC diet; and multiprotease-supplemented NCMP diet reduced the N consumed and excreted ($P < 0.05$) at the end of weeks 29, 33, and 37 when compared to the PC diet (Table 6). Furthermore, total N retained tended to be reduced ($P = 0.057$) at the end of week 33 with reduced dietary AA/CP diets (NC and NCMP) relative to the PC diet. Multiprotease-mediated improvements ($P < 0.05$) in the final N retained in the egg that were equivalent to the PC diet were observed with the NCMP diet at the end of week 37.

Economic performance

Reduced feed costs were noticed ($P < 0.05$) with the reduced protein and amino acid diets (NC and NCMP) at the end of 31, 37, and the overall period from 25 to 37 weeks relative to the PC diet (Table 7). Multiprotease in the NCMP diet increased the overall returns on investment ($P < 0.10$) from 25 to 37 weeks of age.

Table 6
Effects of dietary CP/AA levels and protease supplementation on nitrogen balance of laying hens.

Items	Diets[1]			SEM[2]	P-Value[3]
	PC	NC	NCMP		
Week 29 (grams/hen/day)					
N intake[4]	3.02^{b}	2.67^{a}	2.65^{a}	0.055	<0.001
N excreted	1.48^{b}	1.23^{a}	1.14^{a}	0.045	<0.001
Total N retained[5]	1.54	1.42	1.52	0.039	0.409
N retained in egg[6]	1.04	1.01	1.06	0.009	0.261
N retained in body[7]	0.50	0.42	0.46	0.040	0.481
Week 33 (grams/hen/day)					
N intake	3.08^{b}	2.71^{a}	2.71^{a}	0.064	0.014
N excreted	1.38^{b}	1.20^{a}	1.16^{a}	0.034	0.006
Total N retained	1.68	1.50	1.61	0.032	0.057
N retained as egg	1.09	1.04	1.09	0.012	0.083
N retained as body	0.59	0.46	0.53	0.029	0.155
Week 37 (grams/hen/day)					
N intake	3.17^{b}	2.78^{a}	2.77^{a}	0.059	<0.001
N excreted	1.41^{b}	1.14^{a}	1.12^{a}	0.037	<0.001
Total N retained	1.76	1.64	1.64	0.045	0.464
N retained in egg	1.11^{b}	1.04^{a}	1.14^{b}	0.014	<0.001
N retained in body	0.65	0.60	0.50	0.048	0.469

[a-b)] Means with different superscripts within the same column differ significantly.
[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.
[2] Pooled standard error of the mean.
[3] Statistical significance was determined at $P < 0.05$.
[4] Intake was calculated as follows: Daily hen feed intake in grams × (Diet N/100).
[5] Difference between N intake and excreted.
[6] Calculated as egg mass (g/hen/d) × N concentration in eggs assumed as 1.936 % (Barzegar et al., 2019); 1.936 is derived from the determined protein content of an egg at around 12.10 (Miranda et al., 2015).
[7] Nitrogen retained in the body (g/hen/d) calculated as the difference between total N retained and N retained in the egg.

Table 7
Effects of dietary CP/AA levels and supplemental protease on egg income, feed cost, and return on investment of laying hens.

Items	Diets[1]			SEM[2]	P-Value[3]
	PC	NC	NCMP		
Week 31 (USD)					
Egg income[4]	2.61	2.57	2.66	0.082	0.904
Feed cost[5]	0.95[b]	0.93[a]	0.93[a]	0.003	0.001
Return on investment[6]	1.66	1.64	1.73	0.083	0.904
Week 37 (USD)					
Egg income	2.60	2.44	2.60	0.056	0.596
Feed cost	1.00[b]	0.97[a]	0.97[a]	0.003	0.001
Return on investment	1.73	1.53	1.77	0.055	0.638
Week 25-37 (USD)					
Egg income	2.56	2.44	2.59	0.032	0.142
Feed cost	0.96[b]	0.94[a]	0.95[a]	0.005	0.001
Return on investment	1.60	1.47	1.64	0.032	0.094

[a-b)] Means with different superscripts within the same column differ significantly.
[1] PC, adequate AA/CP; NC, 90 % AA/CP; NCMP, NC supplemented with multiprotease.
[2] Pooled standard error of the mean.
[3] Statistical significance was determined at $P < 0.05$.
[4] Egg income was calculated using hen-day egg production, average egg weight, and the price of an egg over two weeks.
[5] Feed costs were estimated based on local ingredient prices and the average feed intake per hen over two weeks.
[6] Calculated as the difference between egg income and feed cost.

Discussion

Significant quantities of feed protein are known to escape digestion in the avian gastrointestinal tract. This inherent inefficiency varies depending on diet composition and several bird-specific factors including age and strain. To maximize nutrient extraction, the use of supplemental protease as a digestibility-enhancing enzyme in broiler diets has been widely studied, showing potential in achieving nutritional, environmental, and economic targets (Olukosi et al., 2015; Cowieson et al., 2017; Yi et al., 2024). However, its effectiveness in laying hen diets remains largely unexplored. This study examined multiprotease effects on dietary protein and amino acid digestibility, ileal histomorphology, productive performance, egg quality, and nitrogen excretion of Hy-Line Brown layers fed reduced CP/AA diets during their early laying period, from week 25 to 37.

The capacity of supplemental protease to increase protein and amino acid digestibility is established and has been consistently reported in broilers (Ding et al., 2016; Park et al., 2020; Lee et al., 2023) and layers (Chen et al., 2021; Poudel et al., 2023). As expected, protease supplementation improved the digestibility of dietary crude protein, and several amino acids including lysine, methionine, phenylalanine, isoleucine, leucine, glutamine, and tyrosine. Concomitantly, Cho et al. (2020) and Penuela-Sierra et al. (2024) reported similar responses in improved crude protein and amino acid digestibility in broilers using the same multiprotease at a similar dosage (300g/ton) that was utilized in the current study. Improved ileal CP/AA digestibility is explained by the capacity of exogenous protease to supplement endogenous enzymatic protein degradation; reduce endogenous losses (Cowieson and Roos, 2016), upregulate the expression of intestinal amino acid transporters (Park et al., 2020); and mitigate the adverse effects of antinutritional factors such as trypsin and chymotrypsin inhibitors that are abundant in the plant-based poultry diets (Penuela-Sierra et al., 2024).

The current improvements in ileal protein and amino acid digestibility appeared to be mediated by the observed improvements in ileal histomorphological measurements including villus height, width, and absorptive surface area. These morphological changes are indicative of enhanced capacity for nutrient absorption at the ileum (Liu et al., 2013) and could be explained by a protease-mediated limitation in the growth of pathogenic microbes (e.g. *Clostridium perfringens* and *Escherichia coli*) in response to reduced quantity of undigested protein (Elling-Staats et al., 2022; Wealleans et al., 2023a). Furthermore, whereas taurine-conjugated bile salts are involved in the absorption of dietary lipids, higher levels of taurine (the predominant amino acid in avian bile) could have cytotoxic effects and impair gut mucosal growth (Huang et al., 2014; Oketch et al., 2023b). Subsequently, the gut health-modulating effects of protease have also been attributed to reduced jejunal taurine concentration, as has been reported with supplemental *Bacillus licheniformis*- based mono-component protease (Cowieson et al., 2017). Further studies to elucidate the effects of protease on intestinal morphology are necessary.

Several parameters of growth and productive performance including the final body weight, body weight gain, egg production, feed intake, and egg loss percentages were unaffected by dietary treatments. It is established that birds adjust their feed intake based on energy requirements. Presently, hens fed isocaloric diets adjusted their feed intake to meet energy demands, ensuring consistent growth and egg production up to 37 weeks. Notably, feed intake was unaffected by dietary treatments and this observation validates any improvements that could be brought by supplemental protease. Furthermore, the present findings align with previous research, showing that reduced CP/AA diets can sustain egg production when supplemented with crystalline amino acids (Cabezas-Garcia et al., 2022; Heo et al., 2023). Cabezas-Garcia et al. (2022) reported that egg production was maintained for up to 54 weeks with 14 % reduced dietary CP, while Heo et al. (2023) found similar outcomes with 13 % CP reduction up to 32 weeks. In contrast, Poudel et al. (2023) observed reduced egg production with hens fed 85 % of the recommended CP/AA levels up to 49 weeks. The long-term potential of reduced CP/AA diets to achieve persistency in lay (up to 100 weeks of age with 450-500 eggs) remains inconclusive, requiring further investigation beyond the reported periods.

Reducing dietary CP/AA by 10 % negatively impacted egg weight, egg mass, and feed conversion ratio, consistent with previous studies (Dong et al., 2017; Barbosa et al., 2020; Poudel et al., 2023). This effect is attributed to a decreased protein-to-energy ratio, resulting from equal caloric intake at reduced protein levels. Consequently, hens fed the unsupplemented 10 % reduced CP/AA diet may have maintained egg production at the expense of egg weight, resulting in lower egg mass values and higher feed conversion ratios. Notably, the imbalance of lower protein per calorie consumed spares excess energy from lowered amino acid deamination and transamination requirements; and is associated with higher abdominal fat pad weights as commonly observed with low CP/AA diets (Heo et al., 2023; Woyengo et al., 2023). Excessive fat accumulation could decrease egg production and quality in laying hens (Xing et al., 2009; Fouad and El-Senousey et al., 2014). Although multiprotease effect on fat accumulation was not specifically assessed in this study, feed enzymes are known to exert broader influences on metabolism (Cowieson and Roos, 2016). Indeed, Yi et al. (2024) reported increased abundance of *Bacteroidetes* and decrease in *Firmicutes* with an alkaline protease from *Bacillus licheniformis*. These results suggest the potential of protease to reduce fat accumulation through changes in the intestinal microbiome composition and could warrant further investigation.

Consistent with the present study, protease supplementation in reduced CP/AA diets allows for considerable protease-mediated improvements in protein degradation and amino acid uptake (Park et al., 2020; Lee et al., 2023). Such improvements could extend to other constituents within the broader feed-nutrient matrix, including fat (Freitas et al., 2011), energy (Olukosi et al., 2015; Penuela-Sierra et al., 2024), and starch (Kalmendal and Tauson, 2012). These enhancements are expected to translate into performance improvements comparable to nutritionally adequate diets. In agreement with previous reports (Novak et al., 2006; Chen et al., 2021; Poudel et al., 2023), the present study demonstrated that multiprotease improved the egg weights and egg mass values while reducing the feed conversion ratios of the

supplemented hens. Despite being highly heritable, egg weight is also affected by dietary protein and amino acid intake (Ji et al., 2014; Dong et al., 2017; Macelline et al., 2021). The present improvements in amino acid availability and utilization are directly associated with the increased egg weights and subsequently, the higher egg mass and lower feed conversion ratios. Notably, the egg weights and feed conversion efficiency values of the supplemented birds were comparable to the nutritionally adequate diets; and suggest multiprotease capacity to restore performance losses resulting from feeding reduced dietary CP/AA diets.

It was hypothesized that multiprotease modulation of protein digestion and amino acid utilization, as well as its broader effects on other nutrients, could improve the internal egg and eggshell quality. In this study, multiprotease supplementation improved Haugh units, indicating enhanced egg freshness and protein content. Given the improved egg weight and albumen height of the protease-supplemented hens, it is plausible that the observed improvements in the Haugh units are a direct response to the observed multiprotease-mediated improvements in amino acid utilization. Concomitantly, Poudel et al. (2023) reported the capacity of supplemental protease to improve egg weight, Haugh units, and albumen height of supplemented hens. Furthermore, marginally improved yolk color intensity was also observed with supplemental protease. Yolk color, a key sensory attribute that influences consumer preference, is primarily determined by dietary intake of carotenoid-rich ingredients, such as yellow corn and corn gluten meal (Ji et al., 2014; Kim et al., 2019). Given the similarity in corn inclusion levels across all basal diets, the observed increase in yolk color is attributed to probable protease-mediated enhancement of fat-soluble pigment absorption and deposition within the egg yolk. The uptake of xanthophylls is directly associated with the fat absorption process (Zaripheh and Erdman Jr, 2002). Consequently, improved fat digestibility could potentiate improved xanthophyll uptake. Protease could facilitate the mobilization of xanthophylls through improved fat digestibility, as previously reported (Freitas et al., 2011). Further evidence of improved yolk color has been observed with the emulsifying effect of dietary lysolecithin supplementation; and subsequently, potential improvements in fat utilization and uptake of fat-soluble pigments (Wealleans et al., 2023b).

The eggshell is a porous and multi-layered bioceramic composite mainly composed of 96 % polymorphic calcium carbonate in the form of calcite; an organic matrix; and a variety of trace elements (Hincke et al., 2012). The role of calcium as a structural element of the eggshell is established (Li et al., 2018). Trace minerals including Cu, Mn, Zn, and Fe also play an important role as co-enzymes and interact with calcite minerals to improve eggshell strength and thickness (Nys et al., 2018; Zhang et al., 2021). In the current study, improved eggshell-breaking strength is associated with the impact of reducing dietary crude protein. The current improvements suggest the impact of dietary protein levels on mineral digestibility and utilization in laying hens. It is suggested that enhanced eggshell-breaking strength with low protein diets is due to probable improvements in the digestibility and utilization of Ca and trace minerals that are fundamental for the eggshell ultrastructure. Indeed, Dao et al. (2022a) reported improved ileal digestibility of Ca; and Dao et al. (2022b) noticed improved digestibility of trace minerals including Zn, Cu, and Fe with dietary crude protein reduction in broilers. Furthermore, Li et al., (2024) reported the capacity of dietary CP reduction from 21 % to 19 % to potentially improve the utilization of P, Cu, and Mn in broilers.

Despite the complex mechanism behind improved eggshell breaking-strength with low protein diets, reduced dietary protein intake is linked to lowered potential renal acid load (**PRAL**; Scialla and Anderson, 2013), and consequently, a hypocalciuretic effect characterized by increased Ca retention (Cowieson et al., 2020). Furthermore, higher dietary protein intake results in increased hindgut fermentation of undigested protein contents; higher gut pH (around 7 in ceca); and increased proliferation of pathogenic microbes (Elling-Staats et al., 2022; Woyengo et al., 2023). Consequently, reduced dietary CP could stabilize gut pH along with the increased abundance of beneficial microbes including *Lactobacillus* and *Bifidobacterium*; and consequently, a favorable acidic gut environment for the ionization process that is fundamental for mineral absorption. Indeed, increased *Lactobacillus* abundance has been reported with a 7 % reduced dietary protein intake (De Cesare et al., 2019). Improved intestinal microbial balance through the colonization of beneficial intestinal microbiota is also associated with improved eggshell quality (Oketch et al., 2024).

Furthermore, alluding to the wider influence of feed enzymes beyond their primary target nutrient within the nutrient matrix (Cowieson and Roos, 2016), supplemental multiprotease resulted in further improvements in the egg-breaking strength relative to the non-supplemented diets. Improved egg-breaking strength suggests multiprotease influence on mineral absorption and utilization; and is expected to be reflected in the eggshell quality and could translate to reduced egg breakage rates (Cai et al., 2024). One limitation of our study, however, is that the effect of dietary protein levels and supplemental protease on mineral digestibility along with bone measurements were not analyzed. To our knowledge, the impact of dietary protein levels on mineral absorption and utilization in poultry is scant. In addition, the potential impact of the interaction between dietary protein and mineral status on egg quality and bone health of laying hens has not received enough attention in scientific literature and previous results have varied. Poudel et al. (2023) reported marginally reduced eggshell color and thickness with dietary CP reduction. Further investigations into the interaction between mineral status and dietary protein for laying hens are warranted.

It remains a principal focus of the animal industry to improve animal performance, health, and welfare; reduce feed costs; and lower environmental stress due to nutrient excretion. The utilization of low-protein diets to reduce feed costs and N excretion is established (Wang et al., 2017). Given its potential to improve nutrient utilization, supplemental protease in low-protein diets was hypothesized to potentiate extra benefits. In this study, dietary protein reduction and protease supplementation linearly decreased the nitrogen excreted, as previously corroborated (Novak et al., 2006; Heo et al., 2023). Reduced N excretion with low protein diets is largely attributed to reduced N intake (Heo et al., 2023; Lee et al., 2024). Additionally, improved nitrogen retained in the egg was noticed with supplemental multiprotease, relative to the non-supplemented low-protein diet. The current improvements are attributed to the reported protease-mediated improvements in the egg mass. Regarding the economic performance, it was expected that reduced nutrient density would reduce feed costs. Increased nutrient density comes with higher feed costs (de Persio et al., 2015). As expected, significantly reduced feed costs were noticed with the low protein and AA diets when compared to the adequate diet. Additionally, multiprotease supplementation in the reduced CP/AA diet lowered feed costs while improving egg revenue, resulting in improved returns on investment. In general, while reduced nutrient density led to lowered feed costs, further improvements in the overall returns on investment were noticed with multiprotease supplementation.

Conclusively, low CP/AA diets maintained the egg production rate while reducing nitrogen excretion and feed costs. Reduced nitrogen excretion and its potential to reduce environmental pollution supports sustainable poultry farming. Multiprotease modulation of ileal absorptive capacity and protein utilization is linked to improved feed conversion efficiency, heavier egg weights, and the maintained egg quality of the supplemented hens. Higher returns on investment due to improved egg revenue and reduced feed costs were also observed with multiprotease. From a practical point of view, the findings highlight the promising potential of supplemental multiprotease to restore performance losses of laying hens fed reduced CP/AA diets, eliciting both economic and environmental benefits. Further investigation of multiprotease effects beyond the current test period is recommended to ascertain the possibility of any noticeable long-term effects into the mid

to late laying cycles. As the global poultry industry increasingly moves towards low-protein diets, further evaluation of the influence of dietary protein level on mineral digestibility and utilization and the overall effect on bone morphology and mineralization are warranted. Further research on the effects of protease on fat metabolism and accumulation; gut microbiota diversity; mineral digestibility and utilization; intestinal morphology and maturation; and egg quality is also recommended.

Declaration of competing interest

All the listed authors reviewed and approved the submission of the current manuscript to Poultry Science. The authors declare no competing interests.

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References

Aderibigbe, A.S., Wise, T.L., Davis, J.D., Naranjo, V.D., Hess, V., Dozier, W.A., 2024. Effects of reduced crude protein diets while maintaining essential amino acid concentrations on growth performance, nitrogen output, ammonia production, and meat yield. Poult. Sci. 103, 103572.

AOAC International, 2006. Official Methods of Analysis, 18th ed. Rev. 2. AOAC Int., Gaithersburg, MD, USA.

AOAC International, 2007. Official Methods of Analysis, 18th ed. Rev. 2. AOAC Int., Gaithersburg, MD, USA.

Barbosa, S.A.P.V., Correa, G.S.S., Correa, A.B., de Oliveira, C.F.S., Vieira, B.S., de Figueiredo, E.M., Junior, J.G.C., Neto, H.R.L., 2020. Effects of different proteases on commercial laying hens at peak production. Rev. Bras. Zootec. 49, 1–10.

Barekatain, R., Nattrass, G., Tilbrook, A.J., Chousalkar, K., Gilani, S., 2019. Reduced protein diet and amino acid concentration alter intestinal barrier function and performance of broiler chickens with or without synthetic glucocorticoid. Poult. Sci. 98, 3662–3675.

Barzegar, S., Wu, S.B., Noblet, J., Choct, M., Swick, R.A., 2019. Energy efficiency and net energy prediction of feed in laying hens. Poult. Sci. 98, 5746–5758.

Cabezas-Garcia, E.H., Rodríguez-Aguilar, D.E., Afanador-Téllez, G., 2022. Individual egg production of Hy-Line Brown hens during the early laying phase in response to dietary CP levels. Animal 1, 100027.

Cai, P., Liu, S., Tu, Y., Fu, D., Zhang, W., Zhang, X., Zhou, Y., Shan, T., 2024. Effects of different supplemental levels of protease DE200 on the production performance, egg quality, and cecum microflora of laying hens. J. Anim. Sci. 102.

Chen, X., Ma, W., Hu, N., Yan, Y., Zhu, Y., Wang, Z., Jiao, G., Chen, X., 2021. Effects of alkaline protease on the production performance, egg quality, and cecal microbiota of hens during late laying period. Anim. Sci. J. 92, 1–11.

Cho, H.M., Hong, J.S., Kim, Y.B., Nawarathne, S.R., Choi, I., Yi, Y.J., Wu, D., Lee, H., Han, S.E., Nam, K.T., Seoung, E.I., Heo, J.M., 2020. Responses in growth performance and nutrient digestibility to a multi-protease supplementation in amino acid deficient broiler diets. J. Anim Sci. Technol. 62, 840–853.

Cowieson, A.J., Roos, F.F., 2016. Toward optimal value creation through the application of exogenous mono-component protease in the diets of non-ruminants. Anim. Feed Sci. Technol. 221 (331), 340.

Cowieson, A.J., Zaefarian, F., Knap, I., Ravindran, V., 2017. Interactive effects of dietary protein concentration, a mono component exogenous protease and ascorbic acid on broiler performance, nutritional status and gut health. Anim. Prod. Sci. 57, 1058–1068.

Cowieson, A.J., Lu, H.., Ajuwon, K.M., Knap, I., Adeola, O., 2016. Interactive effects of dietary protein source and exogenous protease on growth performance, immune competence and jejunal health of broiler chickens. Anim. Prod. Sci. 57, 251–261.

Cowieson, A.J., Perez-Maldonado, R., Kumar, A., Toghyani, M., 2020. Possible role of available phosphorus in potentiating the use of low-protein diets for broiler chicken production. Poult. Sci. 99, 6954–6963.

Dao, H.T., Moss, A.F., Bradbury, E.J., Swick, R.A., 2022a. Bone measurements of arginine deficient broilers growing under warm temperatures. Trop. Anim. Sci. J. 45, 356–367.

Dao, H.T., Moss, A.F., Bradbury, E.J., Swick, R.A., 2022b. Bone mineralisation status of broilers fed reduced-protein diets supplemented with L-arginine, guanidinoacetic acid and L-citrulline. Anim. Prod. Sci. 62, 539–553.

De Cesare, A., Faria do Valle, I., Sala, C., Sirri, F., Astolfi, A., Castellani, G., Manfreda, G., 2019. Effect of a low protein diet on chicken ceca microbiome and productive performances. Poult. Sci. 98, 3963–3976.

De Cloet, C.A., Maina, A.N., Schulze, H., Bedecarrats, G.Y., Kiarie, E.G., 2023. Egg production, egg quality, organ weight, bone ash and plasma metabolites in 30-week-old Lohmann LSL lite hens fed corn and soybean meal-based diets supplemented with enzymatically treated yeast. Poult. Sci. 102, 102527.

dePersio, S., Utterback, P.L., Utterback, C.W., Rochell, S.J., O'Sullivan, N., Bregendahl, K., Arango, J., Parsons, C.M., Koelkebeck, K.W., 2015. Effects of feeding diets varying in energy and nutrient density to Hy-Line W-36 laying hens on production performance and economics. Poult. Sci. 94, 195–206.

Ding, X.M., Li, D.D., Li, Z.R., Wang, J.P., Zeng, Q.F., Bai, S.P., Su, Z.W., Zhang, K.Y., 2016. Effects of dietary crude protein levels and exogenous protease on performance, nutrient digestibility, trypsin activity and intestinal morphology in broilers. Livest. Sci. 193, 26–31.

Dong, X.Y., Azzam, M.M.M., Zou, X.T., 2017. Effects of dietary threonine supplementation on intestinal barrier function and gut microbiota of laying hens. Poult. Sci. 96, 3654–3663.

Duque-Ramírez, C.F., Javierre, J.A., Peñuela-Sierra, L.M., Diaz-Vargas, M., 2023. Effect of exogenous protease on performance, nutrient digestibility, intestinal histomorphometric, meat quality characteristics, carcass yield in broilers fed low protein diets. Trop. Anim. Health Prod. 55, 190.

Elling-Staats, M.L., Gilbert, M.S., Smidt, H., Kwakkel, R.P., 2022. Caecal protein fermentation in broilers: a review. Worlds. Poult. Sci. J. 78, 103–123.

European Commission, 1999. Council Directive 19999/74/EC laying down minimum standards for the protection of laying hens. Off. J. Eur. Comm. 26, 0053–0057.

Fenton, T.W., Fenton, M., 1979. An improved method for chromic oxide determination in feed and feces. Can. J. Anim. Sci. 59, 631–634.

Fouad, A.M., El-Senousey, H.K., 2014. Nutritional factors affecting abdominal fat deposition in poultry: a review. Asian-Australas. J. Anim. Sci. 27, 1057–1068.

Freitas, D.M., Vieira, S.L., Angel, C.R., Favero, A., Maiorka, A., 2011. Performance and nutrient utilization of broilers fed diets supplemented with a novel mono-component protease. J. Appl. Poult. Res. 20, 322–334.

Heo, J.M., Opapeju, F.O., Pluske, J.R., Kim, J.C., Hampson, D.J., Nyachoti, C.M., 2013. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. J. Anim. Physiol. Anim. Nutr. 97, 207–237.

Heo, Y.J., Park, J.., Kim, Y.B., Kwon, B.Y., Kim, D.H., Song, J.Y., Lee, K.W., 2023. Effects of dietary protein levels on performance, nitrogen excretion, and odor emission of growing pullets and laying hens. Poult. Sci. 102, 102798.

Hincke, M.T., Nys, Y., Gautron, J., Mann, K., Rodriguez-Navarro, A.B., Mckee, M.D., 2012. The eggshell: structure, composition and mineralization. Front. Biosci. 17, 1266–1280.

Huang, C.X., Guo, Y.M., Yuan, J.M., 2014. Dietary taurine impairs intestinal growth and mucosal structure of broiler chickens by increasing toxic bile acid concentrations in the intestine. Poult. Sci. 93, 1475–1483.

Hy-Line Brown Commercial Layers. 2024. Management Guide. Accessed Aug. 29, 2024. Available from https://www.hyline.com/filesimages/Hy-Line-Products/Hy-Line-Product-PDFs/Brown/BRN%20STD%20ENG.pdf.

Ji, F., Fu, S., Ren, B., Wu, S., Zhang, H., Yue, H., Gao, J., Helmbrecht, A., Qi, G., 2014. Evaluation of amino-acid supplemented diets varying in protein levels for laying hens. J. Appl. Poult. Res. 23, 384–392.

Jones, D., 2012. Haugh unit: gold standard of egg quality. USDA ARS. Accessed Sep. 2024. https://www.ars.usda.gov/research/publications/publication/?seqNo115=280843.

Kalmendal, R., Tauson, R., 2012. Effects of a xylanase and pro tease, individually or in combination, and an ionophore coccidiostat on performance, nutrient utilization, and intestinal morphology in broiler chickens fed a wheat-soybean meal-based diet. Poult. Sci. 91, 1387–1393.

Kim, J.H., Lee, H.K., Yang, T.S., Kang, H.K., Kil, D.Y., 2019. Effect of different sources and inclusion levels of dietary fat on productive performance and egg quality in laying hens raised under hot environmental conditions. Asian-Australas. J. Anim. Sci. 32, 1407–1413.

Lee, J., Oh, H., Kim, Y., Song, D., An, J., Chang, S., Go, Y., Cho, H., Lee, B., Kim, W.K., Cho, J., 2023. Effects of exogenous protease on performance, economic evaluation, nutrient digestibility, fecal score, intestinal morphology, blood profile, carcass trait, and meat quality in broilers fed normal diets and diets considered with matrix value. Poult. Sci. 102, 102565.

Lee, J., Yu, M.H., Oketch, E.O., Nawarathne, S.R., Kim, Y.B., Chathuranga, N.C., Maniraguha, V., Sta Cruz, B.G., Seo, E., Park, H., Choi, H., Yu, D.J., Kim, J.K., Heo, J.M., 2024. Effect of dietary protein levels and age on growth performance, total excreta and nitrogen balance of laying hens during the growing phase. Korean J. Agric. Sci. 51, 193–203.

Li, P., Wang, R., Jiao, H., Wang, X., Zhao, J., Lin, H., 2018. Effects of dietary phosphorus level on the expression of calcium and phosphorus transporters in laying hens. Front. Physiol. 9, 627.

Li, X., Wang, C., Li, S., Zhang, L., Liao, X., Lu, L., 2024. Low protein diet influences mineral absorption and utilization in medium-growing yellow-feathered broilers from 1 to 30 days of age. Poult. Sci. 103, 104512.

Liu, S.Y., Selle, P.H., Court, S.G., Cowieson, A.J., 2013. Protease supplementation of sorghum-based broiler diets enhances amino acid digestibility coefficients in four small intestinal sites and accelerates their rates of digestion. Anim. Feed Sci. Technol. 183, 175–183.

Macelline, S.P., Toghyani, M.., Chrystal, P.V., Selle, P.H., Liu, S.Y., 2021. Amino acid requirements for laying hens: a comprehensive review. Poult. Sci. 100, 101036.

Miranda, J.M., Anton, X., Redondo-Valbuena, C., Roca-Saavedra, P., Rodriguez, J.A., Lamas, A., Franco, C.M., Cepeda, A., 2015. Egg and egg-derived foods: effects on human health and use as functional foods. Nutrients 7, 706–729.

Nannoni, E., Buonaiuto, G., Martelli, G., Lizzi, G., Trevisani, G., Garavini, G., Sardi, L., 2022. Influence of increased freedom of movement on welfare and egg laying pattern of hens kept in aviaries. Animals 12, 2307.

Ndazigaruye, G., Kim, D.H., Kang, C.W., Kang, K.R., Joo, Y.-J., Lee, S.R., Lee, K.W., 2019. Effects of low-protein diets and exogenous protease on growth performance, carcass

traits, intestinal morphology, cecal volatile fatty acids and serum parameters in broilers. Animals 9, 226.
Neves, D.P., Banhazi, T.M., Nääs, I.A., 2014. Feeding behaviour of broiler chickens: a review on the biomechanical characteristics. Rev. Bras. Cienc. Avic. 16, 01–16.
Novak, C., Yakout, H.M., Scheideler, S.E., 2006. The effect of dietary protein level and total sulfur amino acid: lysine ratio on egg production parameters and egg yield in Hy-Line W-98 hens. Poult. Sci. 85, 2195–2206.
Nys, Y., Schlegel, P., Durosoy, S., Jondreville, C., Narcy, A., 2018. Adapting trace mineral nutrition of birds for optimising the environment and poultry product quality. Worlds Poult. Sci. J. 74, 225–238.
Oketch, E.O., Kim, Y.B., Yu, M., Cho, H.M., Hong, J.S., Nawarathne, S.R., Wickramasuriya, S.S., Heo, J.M., 2023a. Research note: evaluation of standardized ileal amino acid digestibility in feed ingredients for Pekin ducks. Poult. Sci. 102, 102899.
Oketch, E.O., Lee, J.W., Yu, M., Hong, J.S., Kim, Y.B., Nawarathne, S.R., Chiu, J.W., Heo, J.M., 2022. Physiological responses of broiler chickens fed reduced-energy diets supplemented with emulsifiers. Anim. Biosci. 35, 1929–1939.
Oketch, E.O., Yu, M.., Hong, J.S., Chaturanga, N.C., Seo, E., Lee, H., Hermes, R.G., Smeets, N., Taechavasonyoo, A., Kirwan, S., Rodriguez-Sanchez, R., Heo, J.M., 2024. Laying hen responses to multi-strain Bacillus-based probiotic supplementation from 25 to 37 weeks of age. Anim. Biosci. 37, 1418–1427.
Oketch, E.O., Wickramasuriya, S.S., Oh, S., Choi, J.S., Heo, J.M., 2023b. Physiology of lipid digestion and absorption in poultry: an updated review on the supplementation of exogenous emulsifiers in broiler diets. J. Anim. Physiol. Anim. Nutr. 107, 1429–1443.
Olukosi, O.A., Beeson, L.A., Englyst, K., Romero, L.F., 2015. Effects of exogenous proteases without or with carbohydrases on nutrient digestibility and disappearance of non-starch polysaccharides in broiler chickens. Poult. Sci. 94, 2662–2669.
Park, J.H., Lee, S.I., Kim, I.H., 2020. The effect of protease on growth performance, nutrient digestibility, and expression of growth-related genes and amino acid transporters in broilers. J. Anim. Sci. Technol 62, 614–627.
Peebles, E.D., McDaniel, C.D., 2004. Practical Manual for Understanding the Shell Structure of Broiler Hatching Eggs and Measurements of Their Quality [Bulletin 1130]. Mississippi Agricultural and Forestry Experiment Station, Raymond, MS.
Peñuela-Sierra, L.M., Aragão-Neto, V.L., Lozano-Cruz, P., Mejia-Abaunza, J.N., Ali, M., Cabañas-Ojeda, J., Yang, Y., Alfaro-Wisaquillo, M.C., Quintana-Ospina, G.A., Vasanthakumari, B.L., Wealleans, A., 2024. Multiprotease improves amino acid release in vitro, energy, and nutrient utilization in broilers fed diets varying in crude protein levels. Poult. Sci. 103, 103546.
Poudel, I., Hodge, V.R., Wamsley, K.G.S., Roberson, K.D., Adhikari, P.A., 2023. Effects of protease enzyme supplementation and varying levels of amino acid inclusion on productive performance, egg quality, and amino acid digestibility in laying hens from 30 to 50 weeks of age. Poult. Sci. 102, 102465.
Poudel, I., Hodge, V.R., Wamsley, K.G.S., Roberson, K.D., Adhikari, P.A., 2024. Effects of protease enzyme supplementation and varying levels of amino acid inclusion on productive performance, egg quality, amino acid digestibility and economics of egg production in Hy-Line W-36 laying hen from 50 to 70 weeks of age. J. Appl. Poult. Res., 100452
Qaisrani, S.N., Moquet, P.C., van Krimpen, M.M., Kwakkel, R.P., Verstegen, M.W., Hendriks, W.H., 2014. Protein source and dietary structure influence growth performance, gut morphology, and hindgut fermentation characteristics in broilers. Poult. Sci. 93, 3053–3064.
Ravindran, V., Hew, L.I., Ravindran, G., Bryden, W.L., 2005. Apparent ileal digestibility of amino acids in dietary ingredients for broiler chickens. Anim. Sci. 81, 85–97.
Ravindran, V., Adeola, O., Rodehutscord, M., Kluth, H., van der Klis, J.D., van Eerden, E., Helmbrecht, A., 2017. Determination of ileal digestibility of amino acids in raw materials for broiler chickens - results of collaborative studies and assay recommendations. Anim. Feed Sci. Technol. 225, 62–72.
Rehman, Z.U., Kamran, J., El-Hack, M.E.A., Alagawany, M., Bhatti, S.A., Ahmad, G., Saleem, A., Ullah, Z., Yameen, R.M.K., Ding, C., 2018. Influence of low-protein and low-amino acid diets with different sources of protease on performance, carcasses and nitrogen retention of broiler chickens. Anim. Prod. Sci. 58, 1625–1631.
Scialla, J.J., Anderson, C.A., 2013. Dietary acid load: a novel nutritional target in chronic kidney disease? Adv. Chronic Kidney D. 20, 141–149.
Walk, C.L., Pirgozliev, V., Juntunen, K., Paloheimo, M., Ledoux, D.R., 2018. Evaluation of novel protease enzymes on growth performance and apparent ileal digestibility of amino acids in poultry: enzyme screening. Poult. Sci. 97, 2123–2138.
Wang, J., Yue, H., Wu, S., Zhang, H., Qi, G., 2017. Nutritional modulation of health, egg quality and environmental pollution of the layers. Anim. Nutr. 3, 91–96.
Wealleans, A.L., Ashour, R., Ishmais, A., Al-Amaireh, S., Gonzalez-Sanchez, D., 2023a. Comparative effects of proteases on performance, carcass traits and gut structure of broilers fed diets reduced in protein and amino acids. J. Anim. Sci. Tech. 66, 457–470.
Wealleans, A., Desbruslais, A., Sanchez, D.G., 2023b. Dietary supplementation of lysolecithin compared to a combination of lysolecithin, synthetic emulsifier and monoglycerides, on laying performance and egg quality of laying hens. In: Page 300 in 23rd European Symposium on Poultry Nutrition, 21-24 June 2023. Rimini, Italy.
Woyengo, T.A., Knudsen, K.E.B., Børsting, C.F., 2023. Low-protein diets for broilers: current knowledge and potential strategies to improve performance and health, and to reduce environmental impact. Anim. Feed Sci. Technol. 297, 115574.
Xing, J., Kang, L., Hu, Y., Xu, Q., Zhang, N., Jiang, Y., 2009. Effect of dietary betaine supplementation on mRNA expression and promoter CpG methylation of lipoprotein lipase gene in laying hens. J. Poult. Sci. 46, 224–228.
Yi, W., Liu, Y., Fu, S., Zhuo, J., Wang, J., Shan, T., 2024. Dietary novel alkaline protease from Bacillus licheniformis improves broiler meat nutritional value and modulates intestinal microbiota and metabolites. Anim. Microbiome 6, 1.
Zaripheh, S., Erdman Jr., J.W., 2002. Factors that influence the bioavailablity of xanthophylls. J. Nutr. 132, 531S–534S.
Zhang, K.K., Han, M.M., Dong, Y.Y., Miao, Z.Q., Zhang, J.Z., Song, X.Y., Feng, Y., Li, H.F., Zhang, L.H., Wei, Q.Y., Xu, J.P., Gu, D.C., Li, J.H., 2021. Low levels of organic compound trace elements improve the eggshell quality, antioxidant capacity, immune function, and mineral deposition of aged laying hens. Animal 15, 100401.