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The Improvement of Nutrient Utilization and Performance in Laying Hens Fed By-Product Diets Supplemented with Xylanase and/or Protease Enzymes

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ABSTRACT

Diets formulated for laying hens often contain anti-nutritional factors—such as non-starch polysaccharides (NSPs), trypsin inhibitors, and phytate—that can impede nutrient absorption. To mitigate these effects and enhance feed efficiency, the poultry industry increasingly incorporates exogenous enzymes like xylanase and protease, either separately or synergistically, to improve the digestibility and nutritional value of by-product feed ingredients. This study was conducted to evaluate the effects of xylanase and/or protease enzyme supplementation in laying hen diets on nutrient digestibility and overall bird performance. Four distinct dietary treatments were formulated, including a negative control (NC) diet that did not contain any enzyme supplementation. The remaining three dietary treatments were modifications of the negative control (NC) diet, incorporating either xylanase, protease, or a combination of both enzymes (xylanase + protease). The NC diet was formulated to contain 2,567 kcal/kg of apparent metabolizable energy corrected for nitrogen (AMEn), 0.77% digestible lysine (dLys), and phytase supplementation. Performance trial showed that supplementation of protease and xylanase+protease had higher ($p<0.05$) hen day production (HDP) compared to NC (88.29 and 88.69% vs 83.53%, respectively). AMEn determination study showed that only xylanase improved ($p<0.05$) AMEn, compared to NC (2,754 vs 2,585) kcal/kg. Amino acid digestibility (dAA) study showed that xylanase and xylanase+protease improved ($p<0.05$) dLys by 0.04% and 0.07% respectively. In summary, supplementation of xylanase and protease enhances nutrient utilization and production performance in laying hens fed high by-product diets. The combined use of both enzymes showed the greatest benefits, suggesting that multi-enzyme strategies may be more effective than single-enzyme approaches. These results support the use of enzyme supplementation to enhance feed efficiency and sustainability in poultry production.

Keywords: amino acid; energi; laying hen; protease; xylanase

INTRODUCTION

The diets of laying hens are predominantly composed of cereals and soybean meal, which naturally contain various anti-nutritional factors—including non-starch polysaccharides (NSP), trypsin inhibitors, and phytate—that can impede nutrient absorption. To mitigate these effects, the poultry industry commonly incorporates exogenous enzymes into feed formulations. Xylanase is frequently included in poultry diets, especially when viscous cereals are used, to reduce intestinal viscosity by breaking down soluble non-starch polysaccharides (NSP) such as arabinoxylans and β-glucans (Melo-Durán *et al*., 2021; Choct, 2015). β-Glucans are a type of non-starch polysaccharide found in the cell walls of various organisms, including yeast, bacteria, fungi, and cereal grains (Choi & Kim, 2023).

Recent nutritional studies highlight the potential of enzyme supplementation as a promising strategy to optimize the utilization of by-product feed ingredients, while simultaneously reducing formulation costs by conserving energy and amino acids. These enzymes function by hydrolyzing anti-nutritional factors, thereby enhancing nutrient digestibility and improving the metabolizable energy (ME) and amino acid availability in the diet (Cowieson *et al*., 2019). Xylanase is an enzyme that specifically targets non-starch polysaccharides (NSPs) by cleaving the internal β-xylosidic linkages within the xylan backbone, resulting in the production of xylo-oligosaccharides (XOS) (Morgan *et al*., 2022).

The use of xylanase is widely adopted in commercial laying hen diets as a strategic approach to depolymerize non-starch polysaccharides (NSPs), reduce digesta viscosity, and support intestinal health.

This is particularly relevant in the current production context, which emphasizes reduced dependence on antibiotic growth promoters (Mahmood & Guo, 2020). Xylanase supplementation has been shown to reduce digesta viscosity in the jejunum and ileum, lower caecal pH, and decrease excreta moisture content. Additionally, it enhances energy retention and facilitates the breakdown of both soluble and insoluble fractions of arabinose, glucose, and xylose derived from total non-starch polysaccharides (NSPs) (Nguyen *et al*., 2021).

The inclusion of protease in poultry diets has been shown to enhance amino acid digestibility by hydrolyzing dietary proteins that escape complete degradation by the bird's endogenous gastrointestinal proteolytic enzymes (Woyengo *et al*., 2023). The incorporation of exogenous proteases into poultry feed can enhance protein hydrolysis by complementing the activity of endogenous digestive enzymes, thereby improving the overall digestion and utilization of dietary proteins. (Liu *et al*., 2024). This strategy not only enhances nutrient digestibility and improves feed conversion efficiency but also reduces the moisture and nutrient content of excreta. Moreover, the use of protease contributes to the sustainability of poultry production by decreasing nitrogen excretion into the environment and supporting overall production efficiency (Stefanello *et al*., 2024).

The effectiveness of multi-enzyme combinations remains an area requiring further investigation, as the specific interactions and impacts of different enzyme mixtures are not yet fully understood. Currently, there is limited research assessing the effects of various exogenous enzyme combinations in laying hens fed vegetable protein-based diets supplemented with alternative sources of soybean meal. Conducting comprehensive studies on various enzyme combinations and expanding the existing knowledge is essential for advancing nutritional research and optimizing dietary strategies in laying hens (Huang *et al*., 2024). In such scenarios, accurately predicting the birds' response is essential to enable the formulation of diets with a strategically balanced nutrient profile, thereby ensuring performance outcomes comparable to those achieved with nutritionally complete diets (Cowieson *et al*., 2006).

A thorough understanding of the intrinsic nutritional value of raw materials, along with the expected response to exogenous enzymes—such as matrix values—is highly valuable for feed formulators. A firm grasp of enzyme chemistry makes it possible to draft lower-cost rations that are still unlikely to shortchange the flock on essential nutrients. In the trial described here, 45- to 53-week-old Hy-Line layers received mash feeds spiked with xylanase, protease, or both, all built around a heavy loading of processing by-products. A range of unconventional feedstuffs-palm kernel meal, distillers' dried grains plus solubles, soybean meal, rice bran, and wheat bran-were blended into the formulations. This study was conducted to evaluate the effects of xylanase and/or protease enzyme supplementation in laying hen diets on nutrient digestibility and overall bird performance.

MATERIALS AND METHODS

Ethical Approval

All experimental procedures involving animals were reviewed and approved by the Animal Ethics Committee of the Faculty of Veterinary Medicine, IPB University (Approval No. 053/KEH/SKE/XII/2022), and were carried out in full compliance with the committee's ethical guidelines.

Experimental Design

The experiment was conducted over the period from 45 to 53 weeks of age using a completely randomized design. It began with an 8-week performance trial, followed by assessments of nutrient digestibility, including the determination of nitrogen-corrected apparent metabolizable energy (AMEn) and amino acid digestibility (dAA).

Birds and Housing

This study involved 600 Hy-Line Brown laying hens, aged 45 to 53 weeks with an average body weight of 2.17 kg/bird and hen day production (HDP) 86.91%, housed in a closed-house system at Nugen Research Farm, Lebak Regency, Banten Province, Indonesia. The experimental design included four dietary treatments, each with 10 replicates consisting of 15 birds per replicate. The housing environment was maintained at an average temperature of 27–28 °C throughout the trial. Birds were given ad libitum access to water and their respective experimental diets. A photoperiod of sixteen hours of light per day was provided consistently during the study period.

Dietary treatments

This experiment consisted of 4 dietary treatments. First diet as a negative control (NC) was formulated based on AMEn layer 2567 kcal/kg and dLys 0.77%, the most desirable point as determined by the response surface methodology (RSM) and desirability function (DF) for maximum hen day production (HDP) and egg weight (EW) at a minimum feed convertion rasio (FCR) that results in the lowest feed cost (FC). This diet was supplemented with a combination of xylanase and protease enzymes. The four dietary treatments were established based on the negative control (NC) and supplemented as follows: T1—NC; T2—NC plus xylanase; T3—NC plus protease; and T4—NC with both xylanase and protease. During feed formulation, the NC diet was supplemented with phytase enzyme at a target activity of 300 FTU/kg, ensuring that all treatment diets contained an equivalent phytase level. Xylanase enzyme was supplemented at a rate of 100 g per ton of feed, targeting an activity level of 1,500 EPU/kg. Similarly, the protease enzyme was added at 100 g per ton, aiming for an activity of 5,000 U/kg of feed. For the digestibility trial, diets included 1% bentonite as an inert Acid Insoluble Ash (AIA) marker.

All dietary treatments were produced at a feed mill specializing in the preparation of experimental diets. The formulations included corn, soybean meal, full-fat soybean, distiller's dried grains with solubles (DDGS), palm kernel meal, wheat bran, and rice bran sourced from both imported and local markets. Near-Infrared Spectroscopy (NIRS) was employed to perform proximate analysis of the feed ingredients, which served as the basis for calculating AMEn values according to the methodology outlined in the European Table of Energy Values for Poultry Feedstuffs. AMEn (layer) was calculated as a regression adjustment to AMEn based on the fat level of the ingredient. Total amino acid content was estimated using a regression equation based on protein levels, while digestible amino acids (dAA) were calculated by multiplying total amino acids by their respective availability coefficients. The inclusion rates of supplemental vitamins, minerals (including calcium), and other non-energy components were consistent across all diets. The phytase enzyme was included in all feeds, reflecting industry standard practice. Each diet was prepared individually and offered in mash form. The ingredients and calculated nutrient compositions of the experimental diets are detailed in Table 1.

Data Collection

Performance study. The experimental diets were provided *ad libitum* to the birds from 43 to 53 weeks of age. The initial two weeks of the study served as an adaptation phase, during which no data were collected. Egg weight (EW) was recorded weekly for each replicate. Feed intake (FI) was also measured on a weekly basis for each replicate throughout the experiment. Hen-day production (HDP) per week was calculated by dividing the total number of eggs laid per pen by the number of laying hens per pen, adjusted for mortality (Dörper *et al*., 2023). Egg mass (EM) production was calculated as HDP multiplied by the average EW of the hen (DeLeon *et al*., 2023). FCR was defined as grams of feed consumed per gram of EM (van Eck *et al*., 2024). Feed cost (FC) was calculated as FCR multiplied by the formula price. AMEI and dLI were determined by using daily feed intake (g/bird/day), AMEn $_{(layer)}$ (kcal/kg), and dLys concentration (g/kg) in each dietary treatment (Lyons *et al*., 2023). All birds were weighed one week prior to the start of the experiment and again at its conclusion. Body weight gain was calculated as the difference between the initial and final body weights over the experimental period.

AMEn determination study. At 53 weeks of age, feed consumption and total excreta output were quantitatively recorded for each cage over a continuous four-day period. Daily excreta samples from each cage were pooled, thoroughly homogenized using a blender, and a representative 200 g portion was taken for oven drying. Each diet sample and oven-dried excreta were ground to pass through a 0.75-mm sieve. Dry matter content, nitrogen levels, and gross energy were measured in replicate samples of both diets and excreta.

Amino acid digestibility study. At 53 weeks of age, the birds were humanely euthanized using CO2 asphyxiation before the ileal digesta samples were collected. The ileum was defined as the section of the intestine extending from the yolk sac diverticulum to the ileocecal junction. After collection, the samples were immediately frozen at -20 °C, then oven-dried and ground to pass through a 0.5-mm sieve. Selected portions of both the diet and ileal digesta were analyzed in duplicate to determine dry matter content, amino acid profiles, and acid-insoluble ash (AIA) levels.

Chemical Analysis

Dry matter (DM) of the digesta was determined by oven drying at 105 °C for 3 hours, following the procedures outlined by the Association of Official Analytical Chemists (AOAC, 2019). In contrast, the DM content of the feed was measured by drying at 135 °C for 2 hours, according to AOAC (2019) guidelines. Gross energy was assessed in triplicate using 0.5 g samples with an adiabatic bomb calorimeter (PARR 6200 Calorimeter).

The xylanase enzyme was endo-1,4-β-xylanase (EC 3.2.1.8), originating from a non-GMO strain of *Trichoderma citrinoviride* (DSM 34663) having a minimum activity of 15,000 EPU/g. Xylanase enzyme activity was measured in duplicate at Biovet Laboratory, Huvepharma EOOD, Bulgaria, using a colorimetric assay that quantifies the release of water-soluble dye resulting from the action of endo-1,4-beta-xylanase on a xylan substrate. One Enzyme Unit (EPU) is defined as the amount of enzyme required to release 0.0083 micromoles of reducing sugars, expressed as xylose equivalents, per minute from oat spelt xylan under conditions of pH 4.5 and 50 **°C.**

Protease enzyme activity was measured in duplicate at Nugen Bioscience Laboratory, Indonesia, using a colorimetric assay that quantifies the amount of tyrosine released from the non-specific substrate casein. The released tyrosine reacts with the Folin reagent to form a blue chromophore. One unit of protease activity is defined as the amount of enzyme capable of producing tyrosine equivalent to 1 mg from casein substrate within one minute of reaction at 50 **°C.**

Amino acid concentrations in the diets were quantified following a 24-hour liquid hydrolysis at 110 °C using 6 mol/L HCl, after which amino acid profiles were analyzed with an Amino Acid Analyzer Biochrom 30. Bentonite Acid Insoluble Ash (AIA) content was determined using the gravimetric method as described by the AOAC.

Calculations and Statistical Analysis

Calculation. AMEn was calculated by adjusting for zero nitrogen retention, using a multiplication factor of 8.22 kcal/g, which corresponds to the energy value of nitrogen retained in the body, as described by Alahyari-Shahrasb *et al*. (2017):

AME (kcal/kg) /g feed = GEf − {(AIAf/AIAe) × GEe}

AMEn (kcal/kg) /g feed = AME/g feed – 8.22 {Nf − (AIAf/ AIAe) × Ne}

Where: AIAf = % AIA/g feed; AIAe = % AIA/g feces; GEf = gross energy (MJ) /g feed; GEe = gross energy (MJ) /g feces; Nf = % nitrogen/g feed; Ne = % nitrogen/g feces.

The apparent ileal digestibility of amino acids was determined using the following formula, which incorporates the ratios of acid-insoluble ash (AIA) in the diet and ileal digesta (Garcia *et al*., 2007):

AA Digestibility ileum= [(AA/AIA)f – (AA/AIA)i] / (AA/AIA)f

Table 1. Ingredient composition and calculated nutrient content of experimental diets for laying hens fed corn–soybean meal-based diets supplemented with xylanase and/or protease ingredients[e]

Items	Treatments			
	T1	T2	T3	T4
Ingredient composition (%)				
Yellow corn (L)	47.08	47.08	47.08	47.08
Soybean meal Argentina HP	22.18	22.18	22.18	22.18
DDGS	0.7	0.7	0.7	0.7
Palm kernel meal	0.7	0.7	0.7	0.7
Full-fat soybean	1.26	1.26	1.26	1.26
Rice bran	15.24	15.24	15.24	15.24
Wheat bran	0.7	0.7	0.7	0.7
Limestone medium coarse	6.19	6.19	6.19	6.19
Limes fine coarse	3.19	3.19	3.19	3.19
Bentonite	1.0	1.0	1.0	1.0
Mono-dicalcium phosphate	0.55	0.55	0.55	0.55
L-Lysine HCl 78.8%	0.02	0.02	0.02	0.02
DL-Methionine 99%	0.24	0.24	0.24	0.24
Salt (L)	0.28	0.28	0.28	0.28
Premix [a]	0.26	0.26	0.26	0.26
Others	0.42	0.42	0.42	0.42
Phytase[b]	0.00006	0.00006	0.00006	0.00006
Xylanase[c]	0	0.0001	0	0.0001
Protease[d]	0	0	0.01	0.01
Nutrient composition[e]				
AMEn layer (kcal/kg)	2.567	2.567	2.567	2.567
Crude protein (%)	16.96	16.96	16.96	16.96
Calcium (%)	3.8	3.8	3.8	3.8
Available Phosphorus (%)	0.26	0.26	0.26	0.26
Total amino acids (%)				
Lysine	0.88	0.88	0.88	0.88
Methionine	0.50	0.50	0.50	0.50
Methionine+Cystein	0.78	0.78	0.78	0.78
Threonine	0.63	0.63	0.63	0.63
Tryptophan	0.19	0.19	0.19	0.19
Isoleucine	0.71	0.71	0.71	0.71
Arginine	1.18	1.18	1.18	1.18
Valine	0.80	0.80	0.80	0.80
Digestible amino acids (%)				
dLys	0.77	0.77	0.77	0.77
dMet	0.47	0.47	0.47	0.47
dM+C	0.70	0.70	0.70	0.70
dThr	0.55	0.55	0.55	0.55
dTrp	0.17	0.17	0.17	0.17
dIleu	0.62	0.62	0.62	0.62
dArg	1.03	1.03	1.03	1.03
dVal	0.70	0.70	0.70	0.70

Note : [a]= Premix Provided the following per kilogram of diet: vitamin A, 8,000 IU; vitamin D3, 3,300 IU; vitamin E, 20 IU; vitamin K, 2.5 mg; vitamin B1, 2.5 mg; vitamin B2, 5.5 mg; vitamin B6, 4.0 mg; vitamin B12, 23 mg; niacin, 30 mg; pantothenic acid, 8.0 mg; folic acid, 0.9 mg; biotin, 75 mg; choline, 110 mg; manganese, 90 mg; zinc, 80 mg; iron, 40 mg; copper, 8 mg; iodine, 1.2 mg; selenium, 0.22 mg. [b]=Ronozyme® HiPhos 10,000 FYT, DSM. The enzyme was included at a rate of 60 g/t to supply a guaranteed minimum of 600 FYT=300FTU/kg of feed. [c]= Hostazyme® X 15,000 EPU/g, Huvepharma. The enzyme was included at a rate of 100 g/t to supply a guaranteed minimum 1,500 EPU/kg of feed. [d]= Kingzyme® 50,000 U/g protease, Nugen Bioscience Indonesia. The enzyme was included at 100 g/t to supply a protease of 5,000 U/ kg of feed. [e]= Determined using individual feed analysis result. dLys= digestible lysine, dMet= digestible methionine, dM+C= digestible methionine+cystein, dThr= degistible threonine, dTrp=digestible tryptophan, dIleu=digestible isoleucine, dArg= digestible arginine, dVal= digestible valine. T1 = negative control (NC), T2 = NC+xylanase, T3 = NC+protease, T4 = NC+xylanase+protease

Where:
(AA/AIA)f = ratio AA to AIA marker in the feed
(AA/AIA)i = ratio AA to AIA marker in the ileal digesta

Statistical Analysis

Energy values of the diet and digestibility coefficient are subjected to a one-way ANOVA analysis with a randomized design of treatments using the SAS 9.2 and significance was set $p<0.05$.

RESULTS

Table 2 summarizes the expected and measured activities of xylanase and protease across the various dietary treatments. Xylanase activity reached 1,700 and 1,620 EPU/kg in treatments T2 and T4, respectively, while protease activity peaked at 4,564 and 4,804 U/kg in treatments T3 and T4. Measured xylanase activity was close to or slightly higher than the expected values in the supplemented groups (T2 and T4). In treatments T3 and T4, the measured protease levels were slightly lower than the expected values.

The effects of exogenous enzyme supplementation on AMEn are detailed in Table 3. Birds receiving the xylanase-supplemented diet (T2) exhibited a significant increase ($p<0.05$) in AMEn values compared to the negative control group (T1), reflecting improved energy utilization. Although the protease (T3) and combined xylanase+protease (T4) groups showed numerically higher AMEn values than T1, these differences did not reach statistical significance ($p>0.05$).

The production performance results, summarized in Table 3, indicate that both protease supplementation (T3) and the combined xylanase and protease treatment (T4) significantly enhanced hen day production (HDP) and egg mass (EM) compared to the control group (T1) ($p<0.05$). Digestible lysine intake (dLI) was significantly greater ($p<0.05$) in the combined xylanase and protease group (T4) compared to T1. However, no significant differences were observed among the treatment groups regarding egg weight (EW), feed intake (FI), feed conversion ratio (FCR), feed cost (FC), AMEn intake (AMEI), or body weight gain (Gain).

Apparent ileal amino acid digestibility (Table 4) was significantly enhanced following enzyme supplementation, with the combined treatment group (T4) exhibiting the highest digestibility values for lysine (0.87%), methionine+cystine (0.80%), threonine (0.77%), and arginine (0.93%) ($p<0.05$). Collectively, the combined use of xylanase and protease (T4) consistently yielded favorable outcomes in terms of energy utilization, nutrient digestibility, and production performance.

Table 2. The measurement of enzyme activities in laying hens fed diets supplemented with xylanase and/or protease enzymes

Dietary treatment	Xylanase[a] (EPU/kg of feed)		Protease[b] (U/kg of feed)	
	Expected	Measured	Expected	Measured
T1	-	290	-	265
T2	1,500	1,700	-	282
T3	-	296	5,000	4564
T4	1,500	1,620	5,000	4804

Note: [a]EPU: xylanase units defined as the amount of enzyme which releases 0.0083 micromol of reducing sugars (xylose equivalent) per minute from oat spelt xylan at pH 4.5 and 50°C. [b]U: protease units defined as the amount of enzyme that can produce amino acid equivalent to 1 mg tyrosine from a casein substrate within 1 1-minute reaction at 50 oC. T1= negative control (NC), T2= NC+xylanase, T3= NC+protease, T4= NC+xylanase+protease.

DISCUSSION

This study demonstrates that dietary supplementation with xylanase and protease, administered individually or in combination, markedly enhances nutrient utilization and production performance in laying hens consuming diets rich in by-products. The enzyme activity measurements presented in Table 2 provide valuable insights into enzyme stability and functionality under practical feeding conditions. Xylanase activity in Treatments T1 and T3 was recorded at 290 EPU/kg of feed. Meanwhile, protease activity was measured at 265 U/kg and 282 U/kg of feed in Treatments T1 and T2, respectively.

Table 3. Performance variables, nutrient utilization, and feed cost of laying hens fed diets containing high levels of by-product ingredients supplemented with xylanase and/or protease

Variables	Dietary treatments				p-value
	T1	T2	T3	T4	
AMEn *as is* (%)	2,585±54[b]	2,754±113[a]	2,652±180[ab]	2,716±59[ab]	0.05
HDP (%)	83.53±2.95[b]	87.12±3.3[ab]	88.29±2.39[a]	88.69±2.23[a]	0.006
EW (g)	65.08±2.20[a]	64.14±2.18[a]	65.26±1.63[a]	64.98±1.52[a]	0.693
EM (g)	52.23±5.43[b]	55.96±3.45[ab]	57.61±1.74[a]	57.66±2.29[a]	0.016
FI (g/bird/day)	115.6±6.0[a]	119.3±6.3[a]	122.0±4.3[a]	121.3±3.9[a]	0.081
FCR	2.17±0.08[a]	2.14±0.12[a]	2.13±0.04[a]	2.11±0.10[a]	0.687
FC (IDR/kg egg)	14,525±506[a]	14,457±672[a]	14,346±299[a]	14,271±698[a]	0.827
AMEI (kcal/bird/day)	302.23±19.7[a]	320.23±23.76[a]	316.81±28.69[a]	323.23±7.3[a]	0.206
dLI (g/bird/day)	0.82±0.08[b]	0.88±0.06[ab]	0.85±0.06[b]	0.93±0.04[a]	0.004
Gain (g)	-147±65.4[a]	-63.7±70.2[a]	-91.1±92.1[a]	-62.6±91.8[a]	0.139

Note: AMEn= apparent metabolizable energy corrected for nitrogen, HDP= hen day production, EW= egg weight, EM= egg mass, FI= feed intake, FCR= feed conversion ratio, FC= feed cost, AMEI= AMEn intake, dLI= digestible lysine intake, T1= negative control (NC), T2= NC+xylanase, T3= NC+protease, T4= NC+xylanase+protease. [a,b] Values with different superscripts in the same row differ significantly ($p<0.05$).

Table 4. Apparent ileal digestibility of amino acids in laying hens fed diets containing high levels of by-product ingredients supplemented with xylanase and/or protease

Variables	Dietary treatments				p-value
	T1	T2	T3	T4	
dLys (%)	0.80±0.04^{c}	0.84±0.03^{ab}	0.80±0.03^{bc}	0.87±0.01^{a}	0.01
dMet (%)	0.79±0.05^{a}	0.85±0.05^{a}	0.80±0.04^{a}	0.85±0.04^{a}	0.036
dMet+Cys (%)	0.72±0.02^{bc}	0.79±0.02^{ab}	0.71±0.02^{c}	0.80±0.01^{a}	0.002
dThr (%)	0.65±0.06^{b}	0.71±0.06^{ab}	0.66±0.05^{b}	0.77±0.03^{a}	0.01
dVal (%)	0.64±0.07^{a}	0.68±0.08^{a}	0.66±0.06^{a}	0.73±0.06^{a}	0.108
dArg (%)	0.89±0.01^{b}	0.91±0.01^{ab}	0.90±0.01^{b}	0.93±0.01^{a}	0.000
dIleu (%)	0.68±0.07^{a}	0.71±0.08^{a}	0.68±0.06^{a}	0.75±0.06^{a}	0.257

Note: ^{a,b} Values with different superscripts in the same row differ significantly ($p<0.05$). T1= negative control (NC), T2= NC+xylanase, T3= NC+protease, T4= NC+xylanase+protease, dLys= digestible lysine, dMet= digestible methionine, dMet+Cys= digestible methionine+cystein, dThr= degistible threonine, dVal= digestible valine, dArg= digestible arginine, dIleu=digestible isoleucine.

This value is considered indicative of the activity of endogenous enzymes naturally present within the feed ingredients. The concentration of endogenous xylanase can vary according to the composition and source of the raw feed materials (Melo-Duran *et al*., 2024). Interestingly, the xylanase activity observed in treatments T2 (1,700 EPU/kg) and T4 (1,620 EPU/kg) slightly surpassed the anticipated levels, which may reflect satisfactory thermal stability of the enzyme during feed processing. Contrary to the expected trend, the measured protease activity in Treatments 3 and 4 was lower than anticipated. This reduction may be attributed to diminished thermostability at elevated temperatures, resulting in partial inactivation of the enzyme (Li *et al*., 2015).

The results of this study demonstrated that xylanase supplementation enhanced both apparent metabolizable energy corrected for nitrogen (AMEn) and digestible lysine (dLys) compared to the negative control (NC). Specifically, Treatment 1 (NC) exhibited an AMEn value of 2,585 kcal/kg, while Treatment 2 (NC + xylanase) showed an increased AMEn value of 2,754 kcal/kg. The observed improvement in AMEn value of 169 kcal/kg closely aligns with the metabolizable energy (ME) increase of 154 kcal/kg and exceeds the values reported by Andrade *et al.* (2021) and Whiting *et al*. (2023), which were 150 kcal/kg and 122 kcal/kg of ME, respectively. Primarily, xylanase-mediated hydrolysis of arabinoxylans reduces digesta viscosity, thereby improving nutrient accessibility and absorption (Cowieson & Roos, 2016a). The observed increases in apparent metabolizable energy (AME) following xylanase supplementation are attributed to modifications of fiber fractions and enhanced starch digestibility (Cowieson *et al*., 2015a).

Xylanase catalyzes the degradation of insoluble, highly branched arabinoxylans in the plant cell wall, thereby increasing the accessibility of both endogenous and exogenous enzymes to starch and protein within the endosperm cells (Hassann *et al*., 2019). Supplementation with xylanase has been demonstrated to improve nutrient digestibility and reduce ileal digesta viscosity (Vargas *et al*., 2024). The absence of a significant improvement in apparent metabolizable energy corrected for nitrogen (AMEn) with protease supplementation alone (T3) was anticipated, given that proteins contribute relatively little to the overall energy metabolism in poultry. Nonetheless, the numerical increase observed in the combined enzyme treatment (T4) suggests potential synergistic interactions.

The production performance results revealed an interesting distinction between the effects of energy and protein utilization. The significant increases in hen-day production (4.8–5.2%) and egg mass observed in protease-supplemented treatments (T3 and T4) underscore the critical role of amino acid availability in supporting egg production. This conclusion is further reinforced by the improved digestibility of essential amino acids such as lysine, methionine, and threonine in these treatment groups. The observation that xylanase supplementation alone (Treatment 2) enhanced apparent metabolizable energy corrected for nitrogen (AMEn) without significantly impacting production parameters suggests that energy was not the primary limiting factor for egg production in this trial. Furthermore, the lack of significant effects on feed conversion ratio across all treatments may indicate that the benefits of enzyme supplementation predominantly supported productive performance rather than improvements in feed efficiency under the specific conditions of this study.

In this study, amino acid digestibility was significantly improved in Treatment 4 (NC + xylanase + protease), particularly for lysine (dLys), methionine + cysteine (dMet + Cys), threonine (dThr), and arginine (dArg). The combined supplementation of xylanase and protease demonstrated enhanced efficacy, yielding higher amino acid digestibility values compared to the negative control (Treatment 1). Previous research supports that such synergistic enzyme combinations can improve feed conversion ratios and nutrient utilization, ultimately promoting better growth performance in broiler chickens (Lin *et al*., 2023). The improvement in digestible amino acids (dAA) observed in this study was lower than the +3.7% increase reported by Cowieson and Roos (2016b). Protease supplementation has been shown to enhance ileal dry matter digestibility by approximately 2.3% and ileal digestible energy by 2.7% (Zavelinski *et al*., 2024). Protease supplementation has been shown to enhance ileal amino acid digestibility and frequently improve the digestibility of non-protein nutrients. Proteolysis frequently alters the bulk archi-

tecture of the nutrient matrix in commercial diets, and that disturbance alone can largely explain the observed outcome. Beyond that primary change, a constellation of secondary influences-endogenous enzyme release, the general condition of the gastrointestinal lining, and the energy of the intestinal transporters-appears to fine-tune the beneficial response (Cowieson *et al*., 2015b).

Enzymes are notorious for their compartmentalized specificity, yet the pairing of xylanase and protease reveals an intriguing counter-narrative. The observed synergy suggests that boosting one well-behaved enzyme with another can yield a metabolic bonus far shy of what either could promise alone. By cleaving the xylosic backbones in the plant cell wall, xylanase opens a window for protein and starch to escape into the digestive pool, an act sometimes likened to prying a stubborn lid off a jar. In that freshly exposed space, protease steps in and dismantles any lingering protein scaffolding, thereby scattering leftover fiber into fragments that dissolve more readily in gut fluids. Considered together, the two enzymes turn a recalcitrant feedstuff into a supple mash almost by brute invitation instead of surgical precision (Amerah *et al*., 2016).

It is important to note, however, that the impact of exogenous enzymes on amino acid digestibility has been reported to vary according to the intrinsic digestibility of the diet (Romero *et al*., 2014). The differences in performance quite likely trace back to the unique physical and chemical signatures of each cereal type. When enzymes are added, any lift in energy output usually hinges on how rich the starter ration already is, what substrates happen to be present, the innate digestibility of those ingredients, the overall condition of the digestive tract, and the way the enzyme blend shifts the microbial community living in the gut (Amerah *et al*., 2016). The measured increases in nutrient availability when xylanase is paired with protease highlight a general trend in animal nutrition: blended enzymatic treatments often outperform isolated supplements. Subsequent laboratory and on-farm studies will need to dissect the biochemical pathways responsible for that advantage and translate those insights into user-friendly algorithms that forecast enzyme performance in shifting ingredient matrices.

CONCLUSION

In conclusion, the findings of this study indicate that the supplementation of xylanase and protease significantly enhances nutrient utilization and the productive performance of laying hens fed diets with high levels of by-products. The combined use of both enzymes yielded the most pronounced improvements, highlighting the potential advantages of integrated enzyme strategies over single-enzyme applications in practical feed formulations. These results carry significant implications for sustainable poultry production, especially in contexts where feed ingredient quality varies and the use of agro-industrial by-products is critical for maintaining economic feasibility.

CONFLICT OF INTEREST

Nahrowi and A. Jayanegara serve as editors of the Tropical Animal Science Journal but have no role in the decision to publish this article. The authors declare that they have no conflicts of interest—financial, personal, or otherwise—that could have influenced or be perceived to influence the content of this manuscript.

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