Effect of Microbial Muramidase on Production Performance, Egg Quality, and Digestibility in Laying Hens

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DOI: https://doi.org/10.51584/IJRIAS.2024.907027

Received: 02 July 2024; Revised: 20 July 2024; Accepted: 24 July 2024; Published: 08 August 2024

ABSTRACT

Recently, a new source of muramidase has become available and lack of research has been done to investigate its effect in laying hens. The current study evaluated the effects of different inclusion levels of exogenous microbial muramidase on production performance, egg quality, and digestibility in laying hens. A total of 72 Babcock White laying hens aged 26 weeks were randomly allotted to one of the four dietary treatments (control- basal diet, low- 35,000 LSU (F)/ kg, medium- 45,000 LSU(F)/ kg, and high- 55,000 LSU(F)/ kg) with 6 replicates each, using a randomized complete block design (RCBD) with initial weight as blocking factor. At the end of the study, microbial muramidase did not improve growth, egg production, and economic performance ($P>0.05$). Adding muramidase linearly increased the percent yolk weight and improved yolk color in a linear fashion way, and the low group had higher yolk color compared with the control group ($P<0.05$). Microbial muramidase supplementation quadratically increased dietary AME and AMEn values, and supplemented diets had higher energy values compared with the control diet ($P<0.05$). Inclusion of microbial muramidase quadratically improved the percent ATTD of GE, DM, OM, and ash, supplemented groups had better percent ATTD of GE compared with the control group, whereas medium and high groups had a higher percent ATTD of DM than the control group ($P<0.05$). Percent ATTD of Ca and phosphorus were linearly increased, and the high group had improved ATTD of Ca compared with the control group ($P<0.05$). Therefore, exogenous microbial muramidase can be used as a feed additive in layer nutrition.

Key words: microbial muramidase, egg quality, metabolizable energy, nutrient digestibility

INTRODUCTION

Supplementation of exogenous enzymes to animal feed is a typical practice in modern diet formulation. Enzymes, as feed additives, aid to increase energy and nutrient digestibility and optimize nutritional value on the diet hence improving the overall animal production and product quality. There is a wide array of enzymes available in the market that animal nutritionists can employ to maximize nutrient utilization from various feed ingredients. Well-known examples include phytases, carbohydrases, and proteases which are, extensively used in the animal nutrition industry (Adeola & Cowieson, 2011).

Enzymes, as biological catalysts, play an imperative role in degrading complex dietary constituents that are otherwise indigestible by the animal's endogenous enzymes (Singh & Yadav, 2018). By incorporating poultry diets with exogenous enzymes such as phytases (Abd El-Hack, 2018), glucanases (Zarghi, 2018), and proteases (Philipps-Wiemann, 2018), animal nutritionists aim to maximize the utilization of dietary nutrients, thereby enhancing feed efficiency and diminishing environmental impact associated with undigested nutrients excreta. In addition, beyond their direct benefits to nutrient utilization, exogenous enzymes have been associated to the improvements in the histomorphometry by promoting gut health through improved nutrient absorption and modulation of the intestinal microbiota composition (Oyeagu et al., 2023). These positive outcomes, not only contribute to better growth performance and feed efficiency, but also bolster the immune competence of poultry, resulting in minimizing the incidence of diseases and strengthen overall welfare

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VII July 2024

(Sureshkumar et al., 2023). Furthermore, the economic implications of exogenous enzyme supplementation in poultry production cannot be overlooked. By increasing the availability of nutrients from raw materials, enzymes enable poultry producers to formulate diets that meet the precise nutritional requirements of birds at different stages of growth, ultimately leading to more consistent and higher carcass quality (Yaqoob et al., 2022) and egg products (Abd El-Hack, 2017).

Muramidase, being studied by Maga et al. (2006), Lui et al. (2010), Nyachoti et al. (2012), May et al. (2012), Oliver & Wells (2015), and Park et al. (2021) for its antimicrobial properties in animal production operations, is now increasingly recognized for its potential use as a feed additive. Muramidase is sometimes called lysozyme or 1,4-B-acetylmuramidase. This enzyme is ubiquitous from higher organisms such as mammals and even plants, to lower organisms like bacteria, fungi, and other microbes (Callewaert and Michiels, 2010). In terms of the enzyme classification, lysozyme is under the class of hydrolase, in which this enzyme catalyzes hydrolysis reaction to its substrate peptidoglycan (PGNs), which is a major component of microbial cell fragments present in the digestive tract of animals. It is under subclass carbohydrase which the addition of water molecule cleaves the B-1,4 glycosidic bonds or linkages between N-acetylmuramic acid (Mur2Ac) and N-acetylglucosamine (GlcNac) in the carbohydrate backbone of polysaccharide peptidoglycan (Nelson & Cox, 2004). The supplementation of non-microbial muramidase to degrade peptidoglycans seems to be beneficial to the animal in consideration of the positive effect observed for feed efficiency (May et al., 2012; Oliver & Wells, 2015).

A novel type of enzyme known as microbial muramidase has emerged in the market recently. This enzyme acts on a substrate that is naturally present in the gastrointestinal tract rather than in the feed itself. This enzyme produced by Trichoderma reseei via submerged fermentation and the gene of interest was obtained from Acremonium alcalophilum (Lichtenberg et al., 2017)

Advancements in enzyme technology, such as the emergence of microbial muramidase, has recently gained interest as a potential feed additive in animals. The inclusion of microbial muramidase as feed additive improved gut functionality, health and immunity, energy and nutrient digestibility, and feed efficiency, hence production performance in broilers (Lichtenberg et al., 2017; Boroojeni et al., 2018; Sais et al., 2019; Pirgozliev et al., 2020; Brugaletta et al., 2022); found to improve growth performance of weanling pigs (Schliffka et al. 2019), and improved laying rate, egg mass, feed-to-egg mass (EFSA FEEDAP Panel, 2024). However, lack of research has been done on the effect of microbial muramidase supplementation in egg-type laying hens. Therefore, to address this knowledge gap, the research aimed to evaluate the impact of different inclusion levels of microbial muramidase on growth performance, egg production, egg quality characteristics, and digestibility of energy and nutrients in laying hens.

MATERIALS AND METHODS

Ethical Approval

The study protocol was approved by the Institutional Animal Care and Use Committee of the College of Agriculture and Food Science, University of the Philippines Los Baños (CAFS-UPLB-IACUC Protocol No. 2021-006).

Birds, Housing, and Experimental Design

In the feeding trial, a total of 72 Babcock White laying hens, 25 weeks of age with an average initial body weight of 1.44 ± 0.09 kg were blocked and randomly allotted to four dietary treatments using randomized complete block design (RCBD), in twenty-four cages, six replicates per treatment. The twenty-four cages were divided into six blocks of four cages, and each replicate cage (19'' length x 15'' width x 15'' height) contained three birds. The conventional battery cages were arranged in two sets of a two-tier design in an open-sided house. In each tier (114'' length x 15'' width x 15'' height), had six cages equipped with continuous feeders and water troughs. A plain galvanized iron sheet was carved into pieces and used as feed

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VII July 2024

separators to prevent mixing of dietary treatments within feed troughs. In each feed trough, six feeders (19'' length x 5'' width x 3'' height) were made. Moreover, to ensure that the birds were not able to consume the dietary treatment assigned to the adjoining replicate, a welded wire was used (8" length x 5" width). A lighting program of 16 h of light and 8 h of darkness was employed to stimulate egg laying throughout the trial. The feeding trial lasted for 9 weeks, and it started when the layers were 26 weeks old and reached an average of 85 % HDEP performance, and ended at 34 weeks old. At 34 weeks of age, a digestibility trial was conducted in two-tier battery cages that were located in an open-sided layer house, with individual feeders, nipple drinkers, and dropping collection trays that allowed egg collection as well as the total collection of excreta. A total of 16 were randomly picked from the 72 laying hens used during the feeding trial and, randomly allotted to 4 experimental treatments using a randomized complete block design (RCBD), with 4 replicates per treatment and 1 laying hen per replicate. Thus, 1laying hen from 4 replicate cages was selected randomly and placed in an individual battery cage (19'' length x 15'' width x 15'' height); and not all 6 replicate cages had a representative. The location of cages was the blocking factor in the study. The same lighting program, temperature, and feed formulations in the feeding trial were used in the digestibility study.

Product Description

The Balancius^{TM} with a product code: 5016277 was obtained from DSM Nutritional Products Philippines Inc. This product is a free-flowing granulated preparation of muramidase (EC 3.2.1.17) produced by submerged fermentation of Trichoderma reesei micro-organism. The product had an analyzed microbial muramidase activity minimum of 100,000 LSU (F)/ g. Every single unit of LSU (F) is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labeled peptidoglycan per minute at pH 6.0 and 30 ℃ by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer (Lichtenberg et al., 2017). In addition, the product had an analyzed total viable count in 1 g and Coliform bacteria in 1 g maximum of 50,000 CFU/ g and a maximum of 30 CFU/ g, respectively. The analysis also showed the absence of E. coli and Salmonella in 25 g. The product complied with current FAO/WHO JECFA and FCC recommended purity specifications for food-grade enzymes.

Diets, Ingredients, and Feeding

A total of 4 experimental diets were formulated for this study. Diets were formulated isocaloric and isonitrogenous, and all nutrients were formulated to meet or exceed PHILSAN Feed Reference Standards (2010) nutrient recommendations for egg-type chicken. Specifically, for the Layer 1 phase (18-42 weeks). The three supplemented basal diets used in the study had different inclusion levels of microbial muramidase (Table 1). The birds were fed restrictedly throughout the feeding trial. A maximum of 114 g of feed per bird per day was implemented. The birds were fed twice a day. Once in the morning and once in the afternoon. Access to water was provided all the time throughout the trials. The microbial muramidase inclusion rate in g of product to kg of diet was computed by (Lichtenberg et al., 2017):

$$\mathbf{T_2\ (35{,}000\ LSU(F)\ /\ 1\ kg\ of\ diet} = \frac{\mathbf{1\ g\ of\ product}}{\mathbf{100{,}000\ LSU(F)}} \mathbf{x} \frac{\mathbf{35{,}000\ LSU(F)}}{\mathbf{1\ kg\ of\ diet}} = \frac{\mathbf{0.35\ g\ of\ product}}{\mathbf{1\ kg\ of\ diet}}$$

$$\mathbf{T_3\ (45{,}000\ LSU(F)\ /\ 1\ kg\ of\ diet} = \frac{\mathbf{1\ g\ of\ product}}{\mathbf{100.000\ LSU(F)}} \mathbf{x} \frac{\mathbf{45{,}000\ LSU(F)}}{\mathbf{1\ kg\ of\ diet}} = \frac{\mathbf{0.45\ g\ of\ product}}{\mathbf{1\ kg\ of\ diet}}$$

$$\mathbf{T_4\ (55{,}000\ LSU(F)\ /\ 1\ kg\ of\ diet} = \frac{\mathbf{1\ g\ of\ product}}{\mathbf{100{,}000\ LSU(F)}} \mathbf{x} \frac{\mathbf{55{,}000\ LSU(F)}}{\mathbf{1\ kg\ of\ diet}} = \frac{\mathbf{0.55\ g\ of\ product}}{\mathbf{1\ kg\ of\ diet}}$$

Refined iodized salt was adjusted to have equivalent inclusion rates across all diets. All the feed ingredients used in the study were the following: yellow corn, soybean meal USA, rice bran D1, coconut oil, limestone, MDCP (Sinophos), vitamin (Vitamin, Essential Vet Laboratories Inc.) and mineral premixes (Mineral, Essential Vet Laboratories Inc.), toxin binder (Preventox), DL-methionine (MetAMINO), L-threonine (L-THREONINE, Fufeng), and refined iodized salt. No antibiotic was included in the diets during the experimental period.

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Data Collection

Growth Performance. The 72 laying hens were randomly selected and carefully weighed individually using an electronic kitchen weighing scale (General Master, model: D528) and stratify them according to their initial body weight without undergoing any fasting. Each bird was instilled with a leg band for its identification. A day before the start of the feeding trial (wk 25), the birds were given 114 g of feeds per bird and allowed to clean up it before weighing their initial body weight on the first day of the actual feeding trial (wk 26). Early morning (10:00 AM) on the first day of the feeding trial, the birds were weighed individually using an electronic kitchen weighing scale to determine their initial body weight (g). At the end of the feeding trial (wk 34), the birds weighed again, same time and procedure were followed during the first day of the feeding trial to determine the final body weight (g). Data on the gain in weight was computed by using the formula: Gain in weight (g) = final body weight (g) – initial body weight (g).

Production Performance. Average daily feed intake per bird, the number of eggs per bird, egg weight, hen-day egg production rate, egg mass, and feed conversion per g egg mass were determined. Daily observation and recording of the number of hens per replicate cage, mortality, and egg collection were done. Egg collection was conducted twice a day. Eggs within the replicate were counted and weighed using an electronic kitchen weighing scale (General Master, model: D528) to determine the number of eggs produced and egg weight (g) within the replicate cage. The hen-day egg production (%) was calculated based on the total number of eggs produced during the 9-week experimental period divided by the total number of hen days in the same period multiplied by 100. The average number of eggs laid per bird was calculated by: dividing the total number of eggs laid during the 9-week experimental period by the average number of hen days of the same period. On the other hand, the total egg weight (g) and the total number of eggs laid were used to determine the average egg weight (g). Computed egg mass g per bird per day was obtained by multiplying the data of the average egg weight (g) by hen-day egg production (%). Feed intake (g) was measured every week. Feed intake (g) was computed by: total feed issued (g) – feed refused (g). Data on feed intake (g) was used to calculate daily feed intake (g) and subsequently used to determine the average daily feed intake per bird (g). The average daily feed intake per bird was divided by egg mass g per bird per day to calculate for feed conversion ratio (FCR). The sale value of eggs was determined by: the average no. of eggs produced per hen for 9 weeks x the price of the egg (based on average egg weight, g). Feed consumed (PhP) was calculated by: average feed consumed per bird for 9 weeks or 63 days x feed cost per kg diet. Data on the sale value of eggs (PhP) and feed consumed (PhP) were used to calculate income over feed cost (PhP) (Navasero et al., 2018; and Cuaresma et al., 2021).

Egg Quality Characteristics. One egg per replicate cage was randomly collected at the end of each week. A total of 24 eggs were selected to measure egg weight (g), egg length (mm), egg width (mm), eggshell thickness (mm), eggshell weight with the membrane (g), yolk weight (g), and yolk color; and computed for shape index (%), albumen weight (g), percent albumen, percent yolk, and percent eggshell. The selected eggs were cleaned using a dry cloth to remove any stain or excreta on the eggshell surface before weighing. The eggs were weighed using a Cole-Parmer Symmetry PR-Series precision toploading balance in triplicate, individually to determine egg weight (g). Egg length (mm) and width (mm) were measured using a digital caliper (Lotus LTHT150VCX1 6") and used to calculate shape index (%) by egg width (mm) / egg length (mm) x 100. The eggs were individually cracked using a typical metallic spoon and segregated egg yolk using a stainless egg separator. Moreover, the chalaza was removed by using a stainless tweezer. The yolk and eggshell with inner and outer membranes were placed onto their designated labeled Petri dishes. The DSM Roche yolk color fan was used to evaluate egg yolk color with a scale of 16. The same observer evaluated egg yolk color from the start until the end of the experimental period. The eggshell with inner and outer membrane was washed with tap water to remove the excess albumen and cleaned afterward with a napkin. Air drying was done until a constant weight was achieved. The weights of the Petri dish + egg yolk (g) and Petri dish + dried eggshell with the membrane (g) were determined using Cole-Parmer Symmetry PR-Series precision toploading balance. These data were later on used to determine the egg yolk weight (g) and eggshell weight (g) using a difference method. The albumen weight (g) was calculated by subtracting the egg yolk (g) and eggshell with inner and outer membrane (g) from the total egg weight (g). The data on the egg yolk, eggshell, and albumen weight (g) were used to compute the percent distribution of the total egg weight. Eggshell thickness (mm), with inner and

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outer shell membrane, was determined as average for three different points (bottom, middle, and top), with five readings per site, using a digital caliper (Lotus LTHT150VCX1 6") without cracking the eggshell. Eggshell thickness = (Sharp point thickness + equator point thickness + air cell thickness) / 3 (Dela Cruz et al., 2020)

General Procedures for Digestibility Study

At the start of the experimental period, laying hens were individually weighed using an electronic kitchen weighing scale (General Master, model: D528) and placed in the cages after a 3-h fast. The experimental diets were fed for 10 days ad libitum. The first 7 days served as an adaptation period to the diets and cages. Followed by a 3-day experimental period with feed intake, egg production, and total excreta measured. The total excreta voided from each replicate cage were collected and weighed every 24 h for a total of 3 d by placing clean excreta collection trays under each battery cage. The freshly collected excreta were cleaned immediately by removing feed and visible feather contaminants using a commercial blower and stainless tweezers and subsequently placed in a labeled zip lock resealable plastic storage bag. All the labeled bags were placed in the freezer (-15 °C). All the 3-day excreta samples were thawed and pooled within labeled aluminum containers, oven-dried at 80 °C for 48 h using a Memmert Convection Oven, mixed homogeneously using a commercial blender (Tough Mama, model: NTMBG-6), sieved (0.42-mm screen), and placed in labeled plastic jars and subsequently stored at room temperature prior for analysis. Feed intake was recorded both in adaptation and experimental periods. At the end of the experimental period, laying hens were individually weighed using an electronic kitchen weighing scale (General Master, model: D528) after a 17-h fast. The following formula was used to determine the apparent total tract digestibility (%, ATTD), apparent metabolizable energy, and N-corrected apparent metabolizable energy (AMEn):

$$\textbf{ATTD} = \frac{\textbf{(Nin}-\textbf{Nout)}}{\textbf{Nin}} \textbf{ x 100}$$

$$\textbf{AME (}\text{kcal/kg DM of diet}\textbf{)} = \frac{[(\textbf{Fi x GEf})-(\textbf{E x GEe})]}{\textbf{Fi}}$$

$$\textbf{AMEn (}\text{kcal/kg DM of diet}\textbf{)} = \frac{[\textbf{AME}-[\textbf{8.22 x (Ni}-\textbf{Ne)}]]}{\textbf{Fi}}$$

Where Nin represented the total intake of a certain nutrient in feed, and Nout represented the total excreta output of the homologous nutrient; GEf is the gross energy of diet (kcal/g DM) and GEe is the gross energy of excreta (kcal/g DM); Fi is the feed intake (g DM/d/hen); E is the excreta output (g DM/day/hen) and 8.22 kcal/g of N as the nitrogen correction factor for each gram N retained in the body and eggs; Ni is the nitrogen intake from the diet (g/d) and Ne is the nitrogen output from the excreta (g/d) (Barzegar et al., 2019)

Chemical Analyses

Chemical analyses were conducted by the Animal Nutrition Analytical Services Laboratory at the University of the Philippines Los Baños. The nutrient composition of diet samples was determined by following the procedures of the Official Methods of Analysis of the AOAC (1993) and (Hamilton & Simpson, 1964). Each sample in every chemical analysis was measured in triplicates.

Moisture Determination: The moisture content of the feed sample was determined by oven drying method. Crucibles were placed inside the forced air oven (Shel Lab model: SMO5) and dried overnight at 105 °C until constant weight was achieved. Then the crucibles were placed in a desiccator using tongs for 15-20 minutes to cool down. The cooled crucibles were weighed using an analytical balance (Kern ABJ 220-4NM) one at a time (W_3). During weighing, feed sample was added in the crucible for approximately around 1.0 g (W_1). Placed back to desiccator and then back to the forced air oven (Shel Lab model: SMO5) for drying at 105 °C, overnight until constant weight was obtained. After, the crucibles with feed samples were placed in a desiccator to cool down for 15-20 minutes, then were weighed using an analytical balance (Kern ABJ 220-4NM) (W_2). The percent moisture was calculated by following formula: % moisture = $W_1 – W_2$ / sample weight (g) x 100. Where: W_1 = weight before oven drying; W_2 = weight after oven drying. The percent dry matter was calculated by: 100 – moisture content.

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Ash Determination: the dried feed samples from moisture determination were used in ashing. The crucibles with free moisture feed samples placed in a muffle furnace (Lab Tech model: LEF-103S-1) at 550 °C for at least 4 hours. The appearance of white/gray ash was obtained indicating a complete oxidation of all organic

matter in the samples. It was cooled for 2 hours, then placed back inside the forced air oven (Shel Lab model: SMO5) at 105 °C for 2 hours. The crucibles were placed in a desiccator for 15-20 minutes to cool down. Finally, the crucibles with ash were weighed using an analytical balance (Kern ABJ 220-4NM) (W_4). Percent ash was calculated by: $W_4 – W_3$ / sample weight (g) x 100. Where: W_3 = weight of crucible; W_4 = weight of crucible + ash.

Crude Protein Determination: Crude protein in the feed samples was determined by Kjeldahl method. Weighed approximately of 0.30 g of feed sample and wrapped in a small piece of filter paper. The wrapped feed samples were placed in a forced air oven to dry (Shel Lab model: SMO5) at 105 °C overnight until constant weight was achieved. The dried feed samples were taken in digestion tubes. Then added 5 ml of concentrated H_2SO_4 and 0.5 g Na_2SO_4 of digestion. The tubes were swirled to mix homogenously then placed on heater to start digestion until mixture became clear- blue green in color. The digestion process needed 45-60 minutes to complete (Foss model: Labtech DT208). The digests were cooled and transferred to erlenmeyer flasks. The volume was made up to mark around 125 ml by the addition of distilled water, distillation of the digests were perfromed by an automated Kjeldahl distillation (Foss model Kjeltec^{TM} 8200) unit for 10 minutes. During Kjeldahl distillation,- yellowish color appeared, signifying the presence of NH_4OH. The distillates were then titrated against 0.09848 N HCl solution until the apperance of blue green color appeared. Percent crude protein of the samples was calculated by: % N = (sample titration=blank titration) x meq.N x 0.09828 HCl / sample weight (g) x 100. Where meq. % N= 14/1000 and % CP = % N x 6.25.

Crude Fat Determination: Small filter papers were weighed using analytical balance (Kern ABJ 220-4NM). Approximately 1.0 g of feed samples were placed and wrapped in the pieces of filter papers. The wrapped samples were subjected in oven drying at 105 °C overnight until constant weight was achieved. Dried feed samples were weighed in an analytical balance (Kern ABJ 220-4NM) (W_5). Crude fat was determined by ether extract using Soxhlet method. The moisture free feed samples wrapped in filter papers were placed in the Soxhlet extractor and extracted fat continuously with petroleum ether for 16 hours. Then samples were removed from the extractor and placed on the petri dish to evaporate the solvent. The samples were dried to constant weight at 105 °C, overnight. The samples were cooled in a dessicator and weighed in an analytical balance (Kern ABJ 220-4NM) (W_6). The percent crude fat was determined by: % crude fat = W_5-W_6 / sample weight (g) x 100. Where: W_5 = weight before extraction; W_6 = weight after extraction.

Crude Fiber Determination: Gooch crucibles were weighed in an analytical balance (Kern ABJ 220-4NM) one at a time. Approximately 0.5 g of feed sample was added in a Gooch crucible. All Gooch crucibles installed in the fiber extractor (Auxilab model: Extractor Dosi-Fiber 4 Units) and kept the valve in an off position. The feed samples were rinsed with distilled water thrice. Then samples were added 1/3 full of H_2SO_4 solution. Then opened the cooling circuit and turned on the heating elements, when it started to boil, the power was reduced and left it for 30 minutes. The valves were opened for drainage of H_2SO_4 solution and rinsed with distilled water thrice to completely ensure the removal of acid from the samples. The same procedure was done for alkali digestion by using NaOH solution instead of H_2SO_4. The samples were dried in a forced air oven (Shel Lab model: SMO5) at 105 °C for overnight. Then allowed samples to cool in a dessicator and weighed (W_7). The samples were placed in muffle furnace (Lab Tech model: LEF-103S-1) at 550 °C for 4 hours. The samples were cooled in a dessicator and weighed again (W_8). Calculations were done by using the formula: % crude fiber = $W_7 – W_8$ / sample weight (g) x 100. Where: W_7 = weight after drying; and W_8 = weight after ignition.

Calcium Analysis: The necessary solutions were prepared and standardized as follows: HCl (1+3) was created by mixing 500 ml of concentrated HCl with 1500 ml of distilled water, and concentrated HNO_3 was used accordingly. NH_4OH (1+1) solutions were prepared by mixing 500 ml of NH_4OH with 500 ml of distilled water, and separately adding 20 ml of NH_4OH to 1000 ml of distilled water. A 4.2% $(NH_4)_2C_2O_4$ solution was

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made by dissolving 42 g of $(NH_4)_2C_2O_4$ in hot distilled water and diluting to 1 liter. Methyl red was dissolved in 200 ml of ethanol to form the indicator solution. 0.05 N KMnO4 was prepared by boiling 1.58 grams of KMnO4 in 300 ml of distilled water for 30 minutes, cooling, transferring to a 1 L of volumetric flask, and filling to volume with distilled water, allowing it to stand overnight. For 6N $H2_SO_4$, 167 ml of concentrated H_2SO_4 was mixed with 700 ml of cold distilled water and diluted to 1 liter. $KMnO_4$ was standardized by weighing 0.1 gram of $K_2C_2O_4 \cdot H_2O$ in a 250 ml beaker, adding 100 ml of distilled water and 30 ml of $6NH_2SO_4$, stirring, adding 10 ml of 0.05 N $KMnO_4$, heating to 70 °C, and titrating until a faint pink color appeared. Samples were prepared by weighing 1.0–2.0 grams into a 50 ml porcelain crucible, igniting at 650 °C for 3 hours, adding 40 ml of HCl (1+3) and 5 drops of HNO_3, boiling until dissolved, cooling under a fumehood, filtering into a 100 ml volumetric flask, diluting with distilled water, and thoroughly mixing. For analysis, an aliquot was placed in a 5-10 ml centrifuge tube, 2 drops of methyl red were added, pH was adjusted to 5 with NH_4OH (1+1), color was adjusted with HCl (1+3), heated in boiling water, 10 ml of 4.2% $(NH_4)2C_2O_4$ was added, color was adjusted with HCl (1+3) if necessary, stood overnight, centrifuged at 50,000 RPM for 15 minutes, supernatant was decanted, 25 ml of NH_4OH (1+50) was added, centrifuged and decanted, 10 ml of H_2SO^4 (5 +125) was added, heated to 70°C, and titrated with 0.05 N KMnO4. A blank was run and % Ca was calculated using the formula: (Sample titration – Blank titration) x N KMnO4 x meq. Ca x D.F. / Sample weight where: meq. Ca = 40.08 / 2000 = 0.02004; D.F. = Total volume/Aliquot taken (AOAC, 1955, p. 379; 1984, p. 164).

Phosphorus Analysis: Take 5 ml of original sample solution (a) then it was diluted to 50 ml with distilled water (B). If the sample was high in phosphorus use solution B. If the sample was low in phosphorus, used original solution A. Pipetted 1 ml aliquot from the sample solution and placed in a test tube. Added the following reagents in order: 1 ml H4- molybdate, 1 ml hydroquinone, 1 ml Na_2SO3. Diluted to 10 ml with distilled water. Prepared different concentrations of the standard KH_2PO_4 solution in test tubes. Concentrations to be prepared are 0, 10, 20, 30, 40. And 50 ug P equivalent to 0, 1, 2, 3, 4, and 5 ml standard KH_2PO_4. Remained for 3 minutes. Take the % transmittance reading spectronic 20 at 660 mu. Calculated: % P = ug P in aliquot x D.F. / Sample weight (gas) x 1000 ug/mg where: D.F. total volume / aliquot (AOAC, 1965, p. 654; 1984, p. 167).

The gross energy of the samples were analyzed using bomb calorimetry (Model 6200, Parr Instruments, Moline, IL). Feed samples were weighed approximately 1-2 grams using an analytical balnce (Kern ABJ 220-4NM) and were homogenized for uniformity. Each sample was placed in a compatible sample cup within the bomb, ensuring it was tightly packed and free from external contaminants. The bomb was filled with oxygen and sealed. The ignition wire was carefully inserted, and the bomb was placed into the calorimeter chamber. The combustion process was initiated, and temperature changes were monitored until a constant state was achieved. The resulting temperature rise reflected the heat released by the feed sample's combustion. Gross energy content was calculated using calibration data and recorded temperature changes. Results are reported in units of energy per unit mass (kcal/kg). The excreta samples were also analyzed for gross energy (GE), dry matter (DM), crude protein (CP), crude fat (CF), ash, nitrogen (N), calcium (Ca), and phosphorus using the same methods mentioned above. The microbial muramidase activity in the diets was analyzed by DSM Nutritional Products Philippines Inc. All feed samples were measured in three technical replicates using the method: LYS-101_03E as described by (Lichtenberg et al., 2017).

Statistical Analyses

SAS University Edition Software by SAS Institute (North Carolina, USA) was used to analyze the data. The F-test using two-way ANOVA was used to determine whether there are significant differences among treatments and blocks. Before analysis, assumptions of ANOVA were tested using the following methods: Shapiro-Wilk test for the test of normality of errors. Breusch-Pagan test for the homogeneity of error variances, Durbin-Watson test for the independence of errors, and Tukey's Additivity of Effects test for the independence of treatment and block effects. Otherwise, a non-parametric test using the Friedman Rank Sum Test was performed. Treatment means were separated by Tukey's Honest Significant Different (HSD) post hoc test. Orthogonal polynomial contrasts were used to examine the nature of dependent variables in increasing levels

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of supplemental microbial muramidase. The significant level for each test was set at $P < 0.05$ to detect statistical significance.

RESULTS

Chemical Analyses of Experimental Diets

The nutrient analyses of the diets fed to laying hens during feeding and digestibility trials showed adequate agreement with the calculated values (Table 1). The supplementation of microbial muramidase (Balancius^{TM}) in the diets resulted in an average muramidase activity of 32,740 LSU(F)/kg feed for low, 44,870 LSU(F)/kg feed for medium, and 51,520 LSU(F)/kg feed for high which accounted for 93.54 %, 99.71 %, and 93.67 % of the intended muramidase activity of 35,000, 45,000, and 55,000 LSU(F)/kg feed for low, medium, and high respectively. The analyzed value of muramidase activity in the non-supplemented group was below the limit of quantification of the assay. Thus, the results of analysis on muramidase activity in the experimental diets confirmed the correct addition of the test product within the range of the calculated or expected values $\pm$ 20 %.

Feed Intake and Growth Performance

The average daily feed intake per bird was not different among dietary treatments ($P>0.05$). No statistical difference in the final live weight and gain in weight was also found between dietary groups from week 26 to week 34 of age ($P>0.05$; Table 2).

Production Performance

Overall, the egg production response variables such as the number of eggs per bird, egg weight, hen day egg production, egg mass, and feed conversion ratio were unaffected by the supplementation of microbial muramidase since no statistical differences were found among dietary treatments ($P>0.05$; Table 2).

Economic Performance

For the entire period of 9 weeks, both feeds consumed in kg and PhP, the sale value of eggs (PhP), and income over feed cost (PhP) showed no statistical differences were found among dietary treatments ($P>0.05$; Table 2).

Egg Quality Characteristics

The egg length, egg width, shape index, eggshell weight in grams and percent, and eggshell thickness were clearly unaffected by the supplementation of the different inclusion levels of microbial muramidase in laying hens from week 26 to week 34 of age age ($P>0.05$; Table 3). However, egg length and eggshell thickness had a tendency to improve linearly ($P=0.077$; Table 3) and quadratically ($P=0.097$; Table 3), respectively. No significant differences were found in egg yolk weight (g) and percent egg yolk among dietary treatments, but adding microbial muramidase in layer diets improved percent egg yolk weight, linearly ($P=0.034$; Table 3) and also had a tendency to improve egg yolk weight (g), quadratically ($P=0.099$; Table 3). In addition, supplementation of microbial muramidase had a tendency to improve percent albumen, quadratically ($P=0.065$; Table 3) and albumen weight (g), linearly ($P=0.070$; Table 3). There was at least one treatment that had a different mean in egg yolk color score ($P<0.05$; Table 3). The low inclusion level of microbial muramidase gave a higher egg yolk color score than the control. Overall, for the entire period from week 26 to week 34 of age, egg yolk color was improved in a dose-response linear manner ($P<0.017$; Table 3). Suggested that the optimum inclusion level of microbial muramidase at this period to have a higher egg yolk color score was at 35,000 LSU(F)/ kg.

AME and AMEn Values

The results of the different inclusion levels of microbial muramidase on the total tract apparent metabolizable energy (AME) and nitrogen-corrected apparent metabolizable energy (AMEn) of layer diets in kcal/kg are revealed in (Table 4). Exogenous microbial muramidase supplementation significantly increased dietary AME

and AMEn values in a quadratic manner (P = 0.005; Table 4). Diets supplemented with varying levels of microbial muramidase had higher AME and AMEn values than the non-supplemented diet by 4.76 to 6.33 % and 4.78 to 6.18 %, respectively (P<0.05; Table 4).

Apparent Total Tract Digestibility of Energy and Nutrients

In agreement with AME and AMEn values, the percent apparent total tract digestibility (ATTD, %) of gross energy (GE) responded in the same manner (P = 0.005; Table 5), being 4.37 to 6.15 % significantly higher (P < 0.05; Table 5) than the non-supplemented group when diets were supplemented with microbial muramidase. Inclusion of microbial muramidase in layer diets quadratically improved on percent ATTD of dry matter (DM), organic matter (OM), and ash (P=0.010 and P=0.039, respectively; Table 5), and the medium and high supplemented groups had a higher ATTD of DM compared with the control group (P<0.05; Table 5). The percent ATTD of nitrogen (N), crude protein (CP), and crude fat (CF) did not differ among the dietary treatments (P>0.05; Table 5). In addition, the inclusion of exogenous microbial muramidase in layer diets linearly increased on percent ATTD of calcium (Ca) (P=0.006; Table 5) and phosphorus (P=0.048; Table 5), and laying hens fed with a diet containing 55,000 LSU (F)/ kg improved percent ATTD of Ca compared with those fed with non-supplemented diet (P<0.05).

DISCUSSION

Productive Performance and Egg Quality Characteristics

Feeding trial was conducted in able to give information regarding the acceptance and performance of laying hens when microbial muramidase supplemented in their diets at different inclusion levels. In the present study, the means of the measured response variables in production performance inlcluding, growth, feed efficiency, egg production, and economic performance from week 26 to week 34 of age were not comparable across treatments by the supplementation of microbial muramidase. Hence, rejecting the alternative hypothesis stating at least one inclusion level of microbial muramidase would give a different mean in the response variable. Just recently, a study involving in testing the safety and efficacy of a feed additive consiting of microbial muramidase on laying hens was published by the EFSA-FEEDAP Panel (2024). In their study, supplementation of microbial muramidase did not influence the daily feed intake and final body weight of Lohmann Brown laying hens, which supports the results of our current study. However, laying rate, eggmass, and feed conversion ratio were improved significantly when laying hens fed with diets containing 30,000 LSU(F)/kg compared with the non-supplemented group. These variances might be attributed by several factors including the muramidase level, enzyme activity, nutritive value of the diets, age, breed, management, and the farm's environment. Suggesting further research to explore optimal inclusion rates of microbial muramidase (Balancius^{TM}) for laying hens to effectively enhance their overall productivity in considering those factors. Several studies have also investigated the impact of supplementing microbial muramidase to the diets of broilers. Results shown inconsistency in terms of the effect of muramidase on feed intake, growth response, and feed efficiency. Most studies found that the feed intake of broilers was not comparable among dietary treatments when supplemented with varying levels of microbial muramidase (Boroojeni et al., 2019; Sais et al., 2020; and Pirgozliev et al., 2020). However, in the study of Brugaletta et al., (2022), the daily feed intake of broilers when supplemented with 45,000 LSU (F)/kg inclusion level of muramidase was higher than the non-supplemented group. An improvement in the final live weight of broilers was also reported when the diets supplemented with muramidase (Lichtenberg et al., 2017; Pirgozliev et al., 2020; and Brugaletta et al., 2022). However, opposite results were also found by Boroojeni et al., (2019), and Sais et al., (2020). In the study conducted by Sais et al., (2020), the inclusion of microbial muramidase in broiler diets did not show any difference in FCR. In contrast with the studies of Lichtenberg et al., (2017), Boroojeni et al., (2019), Schliffka et al., (2019), Pirgozliev et al., (2020), and Brugaletta et al., (2022), FCR and European feed efficiency factor of broilers and the FCR of pigs fed with microbial muramidase were improved than the control group. The variability in bird feed intake, growth response, and feed efficiency may be influenced to the different diet formulations, enzyme dose and activity, age, breed, management, environment, and species. Given the diversity in the experimental birds (broilers vs layers) used in evaluating the same origin of lysozyme in vivo,

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VII July 2024

it might be speculated that the mode of action could also be different.

Egg quality has been defined as the properties of an egg that could influence consumer acceptability and preference. In the current study, no effect was observed on the egg quality characteristics of laying hens by microbial muramidase, except for egg yolk color (Table 3). Similarly, other study on the use of muramidase from different origin, e.g. lysozyme (Sindaye et al., 2023), reported no signicant differences were observed on the egg quality characteristics in laying hens. Yolk color is one of the key factors in any consumer survey relating to egg quality. A relatively high score on egg yolk color is essential due to the consumer preference towards egg with golden yellow to orange yolk colors. Although consumer preferences for yolk color are subjective (Gerber, 2006; and Berkhoff et al., 2020). Egg yolk color is derived from yellow carotenoids: lutein, zeaxanthin, and apo-ester, and red carotenoids: canthazanthin, citranaxanthin, and capsanthin. Laying hens cannot synthesize carotenoids de novo and must be derived from the diet (Beardsworth and Hernandez, 2004). Carotenoids and vitamin A are fat soluble compounds that may be used as intestinal absorption biomarker. In the present study, no improvement was observed on the apparent total tract digestibility (ATTD) of crude fat (Table 5.), suggesting that perhaps muramidase did not enhance the absorption rate of the fat soluble vitamin A and provitamin B-carotene, to improve egg yolk color, but rather can be explained due to the increased carotenoids and/or vitamin A concentrations within layers. Although the concentration levels of carotenoids and vitamin A were not analyzed in our study, similar other studies on the use of microbial muramidase in broilers, reported valuable findings in the improvement of carotenoids and vitamin A concentrations (Sais et al., 2020; Goes et al., 2022 and Cho et al., 2024). In the study of Sais et al. (2020), they observed an increase on fat digestibility and plasma vitamn A concentration in birds when supplemented with 35,000 LSU(F)/kg of microbial muramidase. In addition, Goes et al. (2022), revealed total carotenoids concentration (4.38 mg/L) in the whole blood increased in broilers fed with 35,000 LSU(F)/kg compared to the non-supplemented group (3.7 mg/L). However, it was statistically similar to the broilers fed with 25,000 LSU(F)/kg (3.97 mg/L). On a different note, Cho et al. (2024) revealed an improvement in total serum carotenoid in broilers supplemented with microbial muramidase compared to the non-supplemented group. Interestingly, muramidase and precision glycan synergistically enhanced total serum carotenoid. Suggesting to conduct further study in relation to the synergistic effects of using microbial muramidase with other feed additives to optimize overall performance and egg quality. Carotenoids are recognized forming vitamin A precursors through the cleavage and reduction B-carotene. This process endows them with significant antioxidant properties. Bohm et al. (1997) demonstrated that carotenoids can regenerate vitamin E by donating electrons to the alpha-tocopheroxyl radical; and less than 10 % of carotenoids can be converted into Vitamin A (Surai et al., 2000). Brugaletta et al., (2022), reported that the supplementation of microbial muramidase in broiler diets at 45,000 LSU (F)/ kg inclusion level dropped plasmatic hypoxanthine, which is considered as a biomarker for oxidative stress (Muchacka et al., 2018) compared with 25,000 LSU (F)/ kg inclusion level and control. This can be associated with the enrichment of genes linked to glutathione metabolism and reduced peroxisome when a diet containing the same level of microbial muramidase is fed to broilers. Omar et al. (2023) also revealed the significant increased the activites of antioxidant enzymes, total antioxidant activity, catalase, and superoxide dismutase and decreased malondialdehyde, an oxidative stress marker in birds fed with diets supplemented with microbial muramidase. Thus, if these findings are also in parallel to laying chickens, it is safe to assume that the non-supplemented group had lowered egg yolk color score because of its less activated total antioxidant activity and/or reduced vitamin A and carotenoid levels. Thus, suggesting to explore more deeper on the impact of microbial muramidase on carotenoid, vitmain A, and antioxidant levels in laying hen to further analyze its mode of action in improve egg quality.

The linear improvement in the percent egg yolk weight, when supplemented with microbial muramidase, might be explained by the tendency responses shown in linear and quadratic on egg length, eggshell ratio, albumen weight, albumen ratio, and egg yolk weight. The linear improvement on ATTD of Ca and phosphorus could potentially improve the eggshell quality: egg length and percent eggshell weight, since the eggshell is composed primarily of calcium carbonate ($CaCO_3$) and phosphorus. Studies about the phenotypic correlation between external and internal egg quality characteristics showed that eggshell length and percent eggshell weight had negative correlations with percent egg yolk weight. Indicating, an increased improvement in eggshell length and percent weight could lower the percent egg yolk weight. In addition, albumen weight and

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percent weight had negative correlations and egg yolk weight had a positive correlation with percent egg yolk weight. Therefore, the tendency to decrease albumen weight and percent weight, revealed in the current study caused the linear increment in percent egg yolk weight (Zhang et al., 2005; Olawumi and Ogulande, 2008; and Inca, Martinez & Vilchez, 2020).

Digestibility of Energy and Nutrients

Gut health, as a steady state where the microbiome and the intestinal tract of the host, exist in symbiotic equilibrium and where the welfare and performance of the animal is not constrained by the intestinal dysfunction (Celi et al. 2017). Hence, it is safe to say that a healthy and well-functioning gut is the cornerstone for achieving optimum animal performances. In the current study, the supplementation of microbial muramidase in laying hens from week 26 to week 34 of age quadratically improved percent on ATTD of gross energy (GE), dry matter (DM), organic matter (OM), and ash, and linearly improved percent on ATTD of calcium (Ca) and phosphorus, which might suggest an improvement in gut health. To our best knowledge, there is no data available evaluating the effect of microbial muramidase inclusion in layer diets on energy and nutrient digestibility. However, just recently Sindaye et al. (2023) reported the inclusion of different levels of lysozyme in laying hens and showed significant effect on ATTD of crude protein, organic matter, dry matter, and total energy, quadratically where laying hens fed with 300 mg/kg of lysozyme had the highest ATTD of crude protein, organic matter, dry matter, and total energy compared with the non-supplemented group and laying hens fed with 100, and 400 mg/kg of lysozyme. It is also noteworthy that inclusion of microbial muramidase in broiler diets has shown positive impact on the gut health, integrity, microbiota, and antioxidant status as well as intestinal barrier for immunity, which could reportedly lead to effective digestion and absorption capacity. Boroojeni et al. (2019) showed a linear improvement in apparent ileal digestibility (AID) of crude protein (CP), ether extract (EE), and phosphorus in broilers at day 35 age, and the group that was fed diets containing 45,000 LSU(F)/kg had higher AID of CP and EE compared with the control group. The linear improvement in the apparent digestibility of phosphorus is in agreement with the current study, although its values across treatments were lower than in the reported study. This might be because of the different materials, methods, and experimental birds used. In the current study, excreta of layers were used as samples to calculate the ATTD, which underestimates the apparent digestibility, whereas digesta of broilers were used as samples to analyze AID in the reported study, which is a more precise method for calculating apparent digestibility. In addition, exogenous phytase was used in diet formulation in the reported study, which might suggest that microbial muramidase could synergistically enhance phytase activity. Sais et al., (2020), reported that the inclusion of microbial muramidase in the broiler diet improved AID of energy, DM, and OM in broilers. It was also observed that microbial muramidase improved the AID of total fatty acids, MUFAs, PUFAs, and vitamin A in plasma at day 9. Lastly, Pirgozliev et al., (2020), showed a quadratic improvement in DMR, OMR, and NR coefficients and linearly improved FR coefficient in broilers at day 21 when supplemented with microbial muramidase. The quadratic effect on the ATTD of GE and DM of the current study could be explained by the analyzed crude fiber content of the medium diet where it met the crude fiber recommendation of below 4 % (PHILSAN, 2010) whereas the remaining diets exceeded. Generally, high dietary fiber increases digesta viscosity and reduces transit time consequently affecting nutrient absorption and digestibility energy and dry matter (Desbruslais et al., 2021). Just recently, Goes et al. (2022) also revealed better dry matter, fat, and ash digestibility when broilers provided diets with muramidase compared to the non-supplemented group.

The improvements in energy and nutrients digestibility could be explained further due to positive changes in small intestinal morphology, the ability of microbial muramidase to degrade luminal peptidoglycans (PGNs) as well as positive modulation of the intestinal inflammatory response, and the effect on the gut microbiota diversity, composition, and metabolism leading to an optimization of digestive and absorptive functions. Boroojeni et al., (2019), revealed that the supplementation of microbial muramidase did not show any difference in morphometry in the mid-jejunum and mid-ileum of broilers at day 35 of age. However, the inclusion of microbial muramidase improved the immunohistochemical and this is also supported by Sais et al., (2020). However, in the study of Wang et al., (2020), supplementation of microbial muramidase improved duodenal villus height, duodenal villus depth, GC, IEL, and reduced $CD3^+$ T lymphocyte levels of broilers at

ISSN No. 2454-6194 | DOI: 10.51584/IJRIAS |Volume IX Issue VII July 2024

day 26 of age. The variability in the responses may be attributed to the different diet formulations, enzyme dose, age, breed, and type of samples.

It is speculated that the accumulation of bacterial cell wall fragments in the intestinal lumen and on the surface of the gut could impair nutrient digestion and absorption, and the dietary exogenous microbial muramidase might have counteracted this problem. Wang et al., (2020), confirmed that the addition of microbial muramidase did not cause lysis to a test bacterium, while it could degrade purified PGNs. Upon degradation of PGNs, muramyl dipeptide (MDP) was released activating NOD2 receptors, which stimulate the potential host innate immune response by increasing IEL, GC, and reducing $CD3^+$ T cell density, subsequently might alter the host intestinal microbial composition and reduce inflammation in broilers, hence improving gut health leading to improved digestibility and production performance.

The supplementation of non-microbial and microbial muramidase in broiler diets appeared to influence gene enzyme expression and metabolism of the cecal microbiota. Xia et al., (2019), reported that non-microbial muramidase could promote the efficiency of microbiota in the cecal of broilers to utilize non-starch polysaccharides (NSPs) for energy. The anaerobic degradation of NSPs would generate SCFAs such as acetic acid, propionic acid, and butyric acid. The SCFs may enter via the circulatory system, contributing about 3 to 4 % of metabolizable energy (ME) or the energy requirements of birds (Jorgensen et al., 1996; Jamroz et al., 2002). Brugaletta et al., (2022), showed that supplementation of microbial muramidase in broiler diets reduced cecal organic acids due to the decrease in the abundance of genes for starch and sucrose metabolism. Plasmatic energy compounds such as pyruvate, 2-oxoglutarate, and glucose were increased in broilers when supplemented with microbial muramidase, despite the downregulated starch and sucrose metabolism. This might be explained by the abundant Bacteroidetes found in the cecal of broilers when fed with a 45,000 LSU(F)/ kg inclusion level. Bacteroidetes may have increased the production of total short-chain fatty acids (SCFAs), favoring propionate, which might be contributed to hepatic gluconeogenesis, thereby generating these energy compounds (Schwiertz et al., 2009). Therefore, these could be the possible underlying mechanisms explaining the increase in AME and AMEn energy values of diets when supplemented with microbial muramidase compared with non-supplemented diets, and the improvement in percent ATTD in DM, OM, and GE presented in the current study.

The supplementation of microbial muramidase linearly improved on percent ATTD in Ca and phosphorus. This might be due to the promotion of vitamin D concentrations, especially calcitriol, and/or enrichment in gene expression of proteins such as calbidin and transporters. Currently, there is no sufficient evidence that is shown in the results of the study to claim these assumptions. Thus, further examinations are needed to fully understand the potential relationships of microbial muramidase with D vitamins and other contributing factors.

Although supplementation of microbial muramidase in layer diets showed comparable improvement in percent ATTD of GE, DM, and Ca between treatments, it surprisingly did not show any improvement in the productive performances of laying birds at weeks 26 to 34 of age. This may perhaps reflect that the cecal microbiota contributes only 3-4 % of the total energy requirements of chickens. Therefore, the means of the productive performances of laying chickens provided varying microbial muramidase inclusion levels were relatively better but not statistically significant than those of the control.

CONCLUSION

The findings of the study made the following conclusions: (1) the productive performances in layers were not statistically different among dietary treatments when supplemented with exogenous microbial muramidase; (2) addition of exogenous microbial muramidase in the layer diets could improve egg quality characteristics in terms of egg yolk color and percent egg yolk weight, and digestibility of energy, dry matter, organic matter, ash, calcium and phosphorus; (3) layer diets supplemented with exogenous microbial muramidase increased apparent metabolizable energy and N-corrected apparent metabolizable energy implying that the addition of microbial muramidase in formulating high energy density diets is feasible. Therefore, exogenous microbial muramidase can be used as feed additive in layer nutrition, although the biological mechanisms leading to these responses deserve to be investigated further.

CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest. Specifically, there are no financial, personal, or other relationships with any individuals or organizations that could inappropriately influence or bias the content of this manuscript.

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Table 1. Ingredients and nutritional characteristics of the experimental diets from week 26 to week 34 of age (as fed basis).

Dietary Treatment[1]				
Item	**Control**	**Low**	**Medium**	**High**
Ingredients, %				
Yellow corn	53.00	53.00	53.00	53.00
Soybean meal	24.66	24.66	24.66	24.66
Rice bran, D1	9.00	9.00	9.00	9.00
Coconut oil	2.4820	2.4820	2.4820	2.4820
Limestone	8.22	8.22	8.22	8.22
MDCP	1.97	1.97	1.97	1.97
Vitamin premix[2]	0.10	0.10	0.10	0.10
Mineral premix[3]	0.10	0.10	0.10	0.10
Salt	0.2780	0.2430	0.2330	0.2230
Toxin binder	0.05	0.05	0.05	0.05
DL-Methionine	0.100	0.100	0.100	0.100
L- Threonine	0.0400	0.0400	0.0400	0.0400
Muramidase 007	0.00	0.0350	0.0450	0.0550
Calculated composition, %				
ME, kcal/kg	2,800	2,800	2,800	2,800
Muramidase activity, LSU (F)/kg[*]	0	35,000	45,000	55,000
Crude protein (N x 6.25)	17.20	17.20	17.20	17.20
Crude fiber	2.61	2.61	2.61	2.61
Crude fat	6.09	6.09	6.09	6.09
Calcium	3.58	3.58	3.58	3.58
Phosphorus	0.45	0.45	0.45	0.45
Lys, total	0.94	0.94	0.94	0.94
Met, total	0.39	0.39	0.39	0.39
M + C, total	0.69	0.69	0.69	0.69

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Thr, total	0.69	0.69	0.69	0.69
Trp, total	0.21	0.21	0.21	0.21
Analyzed composition, %				
GE, kcal/kg	3,866	3,872	3,922	3,878
Muramidase activity, LSU (F)/kg*	3,627	32,740	44,870	51,520
Dry matter	92.20	92.02	93.38	92.28
Crude protein (N x 6.25)	16.76	16.76	17.04	17.22
Crude fiber	4.49	4.50	3.82	4.11
Crude fat	4.06	4.03	4.29	3.95
Ash	12.28	10.85	11.70	11.81
Calcium	3.78	3.30	3.83	3.71
Phosphorus	0.66	0.81	0.89	0.74

[1]Dietary treatments: Non-supplemented basal diet; low (35,000 LSU (F)/kg); medium (45,000 LSU (F)/kg); high (55,000 LSU (F)/kg).

[2]Provided the following quantities of vitamins per kg of diet: Vitamin A, 50,000,000 IU; vitamin D3, 9,000,000 IU; vitamin E, 200 g; vitamin K3, 9 g; vitamin B1, 9 g; vitamin B2, 22 g; vitamin B6, 14 g; vitamin B12, 0.10 g; niacin, 70 g; biotin, 1.0 g; folic acid, 10 g.

[3]Provided the following quantities of minerals per kg of diet: Iron, 80,000 mg; zinc, 80,000 mg; manganese, 100,000 mg; copper, 10,000 mg; selenium (organic), 12,000 mg; iodine, 300 mg; carrier, 150 mg.

* Every single unit of LSU (F) is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labeled peptidoglycan per minute at pH 6.0 and 30 C by a value that corresponds to the fluorescence of approximately 0.06 nmol fluorescein isothiocyanate isomer.

Table 2. Effect of different inclusion levels of microbial muramidase on productive performance of laying birds from week 26 to week 34 of age (Exp. 1)1.

Item	**Dietary Treatment**[2]					**P-Value**		
						Treatment	Linear	Quadratic
Average daily feed intake, g/bird	**Control**	**Low**	**Medium**	**High**	**SEM**[3]	0.54	0.574	0.347
	106.70	106.00	103.20	105.90	1.746			
Final body weight, g/bird	1,467	1,456	1,488	1,467	16.655	0.47	0.942	0.652
Gain in weight, g/bird	25.06	37.94	33.81	33.89	11.28	0.87	0.486	0.852
Number of eggs, bird	56.72	59.28	55.85	59.51	1.417	0.22	0.530	0.821
Egg weight, g	55.95	54.78	55.45	55.30	0.661	0.67	0.357	0.992
Hen day egg production, %	90.04	94.09	88.65	94.46	2.249	0.22	0.530	0.821

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Egg mass, g/bird/day	50.38	51.55	49.15	52.24	1.251	0.35	0.937	0.830
Feed conversion ratio, g feed/g egg	2.126	2.056	2.106	2.027	0.043	0.38	0.472	0.547
Sale value of eggs[4], PhP	351.90	364.53	360.45	368.96	22.06	0.52	0.43	0.44
Feed consumed[5], kg	6.72	6.68	6.50	6.67	0.36	0.54	0.55	0.67
Feed consumed, PhP	195.27	196.04	191.54	197.01	7.73	0.66	0.83	0.67
IOFC[6], PhP	156.63	168.49	168.91	171.95	19.27	0.55	0.37	0.32

[1]Means of 6 replicates (3 laying birds each).

[2]Control: non-supplemented basal diet, low (35,000 LSU (F)/kg), medium (45,000 LSU (F)/kg), high (55,000 LSU (F)/kg)

[3]Standard error of the mean

[4]Suggested price per egg is based on Silliman Farm. The price per fresh egg depends on its weight. PhP 6.00 (50.1 to 55 g- small), PhP 6.30 (55.1 to 60 g- medium), PhP 6.66 (60.1 to 65 g- large), PhP 7.33 (65.1 to 72.5 g- extra-large), PhP 8.00 (72.6 g & above- jumbo).

[5]Average feed consumed per bird for 9 weeks and diet cost per kg is PhP 29.05 (control), PhP 29.36 (low), PhP 29.45 (medium), PhP 29.54 (high).

[6]Income over feed cost

Table 3. Effect of different inclusion levels of microbial muramidase on egg quality characteristics of laying birds from week 26 to week 34 of age (Exp. 1)[1].

Item	**Dietary Treatment**[2]					**P-Value**		
						Treatment	Linear	Quadratic
Egg length, mm	**Control**	**Low**	**Medium**	**High**	**SEM**[3]	0.21	0.077	0.498
	55.96	55.27	55.27	55.87	0.23			
Egg width, mm	42.61	42.44	42.48	42.57	0.20	0.92	0.507	0.977
Shape index, %	76.19	76.81	76.25	76.22	0.42	0.50	0.321	0.538
Eggshell weight, g	5.47	5.29	5.52	5.49	0.09	0.29	0.205	0.177
Eggshell weight, %	9.57	9.44	9.79	9.66	0.10	0.15	0.591	0.055
Eggshell thickness4, mm	0.3916	0.3961	0.4004	0.3976	0.003	0.10	0.222	0.097
Egg yolk color	6.998b	7.742a	7.112ab	7.370ab	0.16	0.025	0.017	0.455
Egg yolk weight, g	12.90	13.16	13.53	13.27	0.19	0.18	0.277	0.099

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Egg yolk weight, %	22.57	23.56	23.98	23.32	0.35	0.20	0.034	0.116
Albumen weight, g	38.87	37.51	37.41	38.17	0.57	0.28	0.070	0.492
Albumen weight, %	67.86	67.01	66.24	67.03	0.39	0.06	0.081	0.065

[1]Means of 6 replicates (1 egg each).

[2]Control: non-supplemented basal diet, low (35,000 LSU (F)/kg), medium (45,000 LSU (F)/kg), high (55,000 LSU (F)/kg)

[3]Standard error of mean

[4]Eggshell with outer and inner membrane samples were measured to determine eggshell thickness (mm).

[a,b,c]Different superscripts within lines indicate levels of significance at $P < 0.05$.

Table 4. Effect of different inclusion levels of microbial muramidase on apparent metabolizable energy and N-corrected apparent metabolizable energy of layer diets (Exp. 2)[1].

Item	**Dietary Treatment**[2]					**P-Value**		
						Treatment	Linear	Quadratic
AME, kcal/kg	**Control**	**Low**	**Medium**	**High**	**SEM**[3]	0.003	0.009	0.005
	3,110[b]	3,258[a]	3,307[a]	3,288[a]	28.35			
AMEn, kcal/kg	2,993[b]	3,136[a]	3,178[a]	3,159[a]	24.57	0.002	0.006	0.005

[1]Means of 4 replicates (1 laying bird each).

[2]Control: non-supplemented basal diet, low (35,000 LSU (F)/kg), medium (45,000 LSU (F)/kg), high (55,000 LSU (F)/kg)

[3]Standard error of mean

[a,b,c]Different superscripts within lines indicate levels of significance at $P < 0.05$.

Table 5. Effect of different inclusion levels of microbial muramidase on apparent total tract digestibility (ATTD, %) of energy and nutrients of laying birds at week 34 of age (Exp. 2)[1].

Item	**Dietary Treatment**[2]					**P-Value**		
						Treatment	Linear	Quadratic
Gross energy	**Control**	**Low**	**Medium**	**High**	**SEM**[3]	0.004	0.014	0.005
	74.18[b]	77.42[a]	78.74[a]	78.25[a]	0.676			
Dry matter	70.88[b]	73.91[ab]	75.54[a]	74.83[a]	0.012	0.016	0.033	0.010
Organic matter	42.53	41.45	37.78	36.16	2.082	0.173	0.870	0.039

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Ash	57.47	58.55	62.21	63.84	2.082	0.173	0.870	0.039
Nitrogen	48.85	50.93	53.87	52.67	2.310	0.486	0.461	0.144
Crude protein	48.28	50.95	53.88	52.67	2.305	0.395	0.347	0.108
Crude fat	80.62	81.37	82.21	81.04	2.191	0.961	0.798	0.833
Calcium	71.74[b]	73.58[ab]	78.13[ab]	80.09[a]	1.975	0.050	0.006	0.974
Phosphorus	24.99	37.61	40.97	30.04	4.367	0.060	0.048	0.414

[1]Means of 4 replicates (1 laying bird each).

[2]Control: non-supplemented basal diet, low (35,000 LSU (F)/kg), medium (45,000 LSU (F)/kg), high (55,000 LSU (F)/kg)

[3]Standard error of mean

[a,b,c]Different superscripts within lines indicate levels of significance at $P < 0.05$