# Evaluation of a microbial muramidase supplementation on growth performance, apparent ileal digestibility, and intestinal histology of broiler chickens

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**ABSTRACT** The current study evaluated the effects of different inclusion levels of microbial muramidase (Muramidase 007, DSM Nutritional Products) on gastrointestinal functionality by determination of apparent ileal digestibility (AID) of nutrients, investigation of intestinal histomorphology, and quantification of resulting growth performance. Four maize-wheat-soybean experimental diets were produced without (**C**) and with different dosages of muramidase: low (L, 25,000 LSU(F)/kg), medium (M, 35,000 LSU(F)/kg), and high (H, 45,000 LSU(F)/kg); diets were fed to broilers for 35 d. At the end of the experiment, AID of ether extract (EE), crude protein (CP), Ca, and P were determined and samples of the mid-jejunum and -ileum were collected for histomorphological observations. Data were subjected to ANOVA analysis using the GLM procedure. Orthogonal polynomial contrasts were used to assess linear and quadratic effects of different levels of the muramidase. At the end of the trial, Muramidase 007 supplementation linearly increased body weight gain and decreased feed conversion ratio (FCR) ($P \leq 0.05$). Adding the muramidase to broiler diets also linearly increased the European poultry efficiency factor ($P \leq 0.05$). Inclusion of the muramidase in broiler diets linearly increased AID of CP, EE, and P ($P \leq 0.05$), and the H group had a higher AID of EE and CP compared to C group ($P \leq 0.05$). Microbial muramidase supplementation linearly increased ileal villus length to crypt depth ratio and decreased the number of ileal CD45 cells ($P \leq 0.05$). Broilers fed M and H diets had fewer number of CD45 cells in the ileum compared to those in C group ($P \leq 0.05$). In conclusion, the results of the present study demonstrated that inclusion of the microbial muramidase in broiler diets could increase AID of key nutrients and improve growth performance in broilers. Adding microbial muramidase to broiler diets can therefore be considered as an interesting prospect to improve gastrointestinal functionality. Biological mechanisms causing these improvements need to be studied further.

**Key words:** lysozyme, peptidoglycans, nutrient availability, feed efficiency, microbial muramidase

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## INTRODUCTION

An effective functionality of the gastrointestinal (**GI**) tract and its health are key determinants in achieving feed efficiency, animal welfare, and sustainability (Celi et al., 2017). Peptidoglycans (**PGNs**) are components forming the cell wall of bacteria, and considered as conserved products of bacterial metabolism and activity in the GI tract. While there is a massive turnover of PGNs from the bacterial cell wall fragments in the gut during the natural bacterial cell wall recycling, it has been reported that PGNs can have a pro-inflammatory impact on the GI tract of host (Linskens et al., 2001; Kyburz et al., 2003; Mayer, 2012; Wheeler et al., 2014). Muramidase (EC 3.2.1.17), also known as lysozyme or N-acetylmuramidase, is a glycosyl hydrolytic enzyme, which cleaves the $\beta$-1, 4 glycosidic linkages between N-acetylmuramic acid and N-acetyl glucosamine in the carbohydrate backbone of bacterial PGNs. Main PGNs breakdown products are muropeptides that have been reported to modulate inflammatory response in the GI tract of different species. However, the magnitude and type of inflammatory responses modulated by muropeptides depend on the species and cell type (Traub et al., 2006). Muramidases are ubiquitous. The most known muramidase is the one abundantly found in hen egg white (**HEW**), but various muramidases can be found in different types of animal secretions, plants, or microorganisms (Lichtenberg et al., 2017). Studies on pig and poultry reported the beneficial impacts of

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feeding HEW lysozyme on the gut microbiota, including a reduction in pathogenic bacteria e.g., *Clostridium perfringens*, *Escherichia coli*, etc., on the GI tract antioxidant status, including an increase in gene expression of intestinal glutathione peroxidase, and on the GI tract nonspecific immunity, including an increase in gene expression of intestinal interferon-gamma, inteleukin-10 and 18 (Liu et al., 2010; Oliver and Wells, 2015; Long et al., 2016; Abdel-Latif et al., 2017; Gong et al., 2017; Ma et al., 2017). Given the observed impacts of muramidase in poultry and pig nutrition, it can be hypothesized that inclusion of microbial muramidase in broiler diets may improve GI functionality, which finally can lead to better nutrient digestibility and absorption and higher growth performance. Recently, it has been demonstrated that the supplementation of a dietary microbial muramidase in broilers diets improved growth performance and increased the number of *Lactobacillus* spp. in the cecum of broilers (Lichtenberg et al., 2017).

The present study aimed to investigate the impacts of different inclusion levels of microbial muramidase on GI functionality by measuring apparent ileal digestibility (**AID**) of nutrients, examination of intestinal histology, and quantification of resulting animal growth and feed efficiency.

## MATERIALS AND METHODS

### Animals and Experimental Design

The animal trial was conducted in accordance with the Animal Welfare Act of Germany approved by the local state office of occupational health and technical safety (Landesamt für Gesundheit und Soziales, LaGeSo, no. A 0100/13).

Four starter (day 1 to 21) and 4 grower (day 22 to 35) maize-wheat-soybean diets were produced without (**C**) and with different levels of Muramidase 007 (microbial muramidase); low (**L**, 25,000 LSU(F)/kg), medium (**M**, 35,000 LSU(F)/kg), and high (**H**, 45,000 LSU(F)/kg). The tested muramidase product was included as dry commercial formulated form, and it had an analyzed muramidase activity of 65,500 LSU(F)/g product. No antibiotic was supplemented during the experimental period. The diets were isocaloric and isonitrogenous for each feeding phase and meet or exceed the recommendations of the Society of Nutritional Physiology (GfE, 1999). The grower diets contained 3 g titanium dioxide ($TiO_2$) per kg feed (Sigma Aldrich, St. Louis, MO) as an indigestible marker to allow for the determination of AID of nutrients. The compositions of the starter and grower diets are shown in Tables 1 and 2, respectively.

A total of 960 one-day-old male broiler chickens (Cobb) were obtained from a local hatchery (Cobb Germany Avimex GmbH) and were randomly allocated to 48 pens (3.1 $m^2$) with bedding of softwood shaving such that there were 240 birds per treatment, 20 chickens per replicate pen, and 12 replicate pens per treatment.

The temperature was 33°C for the first 7 d of the experiment, after which the temperature was gradually reduced by 3°C per week until reaching 22°C. The artificial light (45 lux) during the first 4 d of age was provided for 24 h continuously. From day 5 of age onwards, the lighting regime consisted of an 18-h light and 6-h dark cycle. The animals had ad libitum access to feed (mash form) and water throughout the experiment.

### Performance Measurements

Body weights of the chicks and their feed intakes (**FI**) were recorded weekly, and body weight gain (**BWG**), FI, and feed conversion ratio (**FCR**) for day 7, 21, and 35 were calculated. The overall European poultry efficiency factor (**EPEF**) was calculated as follows:

$$\text{EPEF} = (\text{Average daily weight gain} \times \text{survival rate}) \div \text{feedconversion ratio} \times 10$$

The data are presented in Table 3.

### Nutrient Digestibility

At the end of the experiment, 3 birds per pen were randomly selected, the ileal digesta was collected from the posterior half between Meckel's diverticulum and 3 cm cranial to the ostium ileocecale for AID determinations. The digesta of all birds within each pen was pooled, immediately frozen (−20°C) and stored until further analysis (ether extract [**EE**], crude protein [**CP**], Ca, and P). The pooled digesta was freeze-dried before chemical analysis.

The following formula was used for AID calculation:

$$\left[\text{AID}\,(\%) = 100 - \frac{\%\ \text{Marker in feed}}{\%\ \text{Marker in the ileum}} \times \frac{\%\ \text{Nutrient in the ileum}}{\%\ \text{Nutrient in feed}} \times 100\right]$$

The AID of nutrients is shown in Table 4.

### Intestinal histology

At the end of the experiment, intestinal samples were also taken for histomorphological studies. For this purpose, 8 pens out of 12 were randomly selected and samples from mid-jejunum and -ileum of 1 broiler chicken (with a body weight close to the mean body weight of the corresponding pen) per pen were taken. The samples were rinsed with Ringer's solution and cut into 3 pieces each. The samples were placed in histology cassettes and immediately fixed for 24 h in a 4% phosphate-buffered formaldehyde solution at room

**Table 1.** Ingredients and nutritional characteristics of the starter diets from day 1 to 21 of age (as is).

| Treatment groups[1] | | Control | Low | Medium | High |
|---|---|---|---|---|---|
| Ingredients | | | | | |
| Maize | g/kg | 319.7 | 319.7 | 319.7 | 319.7 |
| Soybean meal (CP 47%) | g/kg | 333.1 | 333.1 | 333.1 | 333.1 |
| Wheat | g/kg | 252 | 252 | 252 | 252 |
| Soybean oil | g/kg | 52 | 52 | 52 | 52 |
| Limestone | g/kg | 10.3 | 9.9 | 9.8 | 9.6 |
| Monocalcium-phosphate | g/kg | 15 | 15 | 15 | 15 |
| Premix[2] | g/kg | 12 | 12 | 12 | 12 |
| DL-Methionine | g/kg | 3.1 | 3.1 | 3.1 | 3.1 |
| L-Lysine HCL | g/kg | 1.9 | 1.9 | 1.9 | 1.9 |
| L-Threonine | g/kg | 0.9 | 0.9 | 0.9 | 0.9 |
| Muramidase 007 | g/kg | 0 | 0.382 | 0.534 | 0.687 |
| Analyzed nutrient composition | | | | | |
| $AME_n$[3] | MJ/kg | 12.42 | 12.42 | 12.42 | 12.42 |
| Crude protein | g/kg | 219.0 | 218.9 | 219.3 | 218.7 |
| Crude fiber | g/kg | 27.1 | 26.7 | 26.9 | 26.5 |
| Ether extract | g/kg | 74.3 | 75.1 | 74.7 | 74.8 |
| Starch | g/kg | 367.7 | 368.3 | 367.4 | 366.9 |
| Sugars | g/kg | 43.2 | 42.7 | 42.9 | 43.4 |
| Ca | g/kg | 9.3 | 9.3 | 9.2 | 9.2 |
| P (total) | g/kg | 7.4 | 7.5 | 7.4 | 7.3 |
| Na | g/kg | 1.8 | 1.7 | 1.8 | 1.7 |
| Xylanase | BXU[4]/kg | 16,112 | 16,208 | 16,088 | 16,111 |
| Phytase | FTU[5]/kg | 691 | 684 | 676 | 680 |
| Muramidase | LSU(F)[6]/kg | 1863 | 25,067 | 30,305 | 39,833 |

[1]Control: control diet, low (25,000 LSU(F)/kg muramidase), medium (35,000 LSU(F)/kg muramidase), high (45,000 LSU(F)/kg muramidase).

[2]Contents per kg Premix: 600,000 I.U. Vit. A (acetate); 120,000 I.U. Vit. $D_3$; 6,000 mg Vit. E ($\alpha$-tocopherole acetate); 200 mg Vit. $K_3$ (MSB); 250 mg Vit. $B_1$ (mononitrate); 420 mg Vit. $B_2$ (cryst. riboflavin); 300 mg Vit. $B_6$ (pyridoxin-HCl); 1,500 $\mu$g Vit. $B_{12}$; 3,000 mg niacin (niacinamide); 12,500 $\mu$g biotin (commercial, feed grade); 100 mg folic acid (cryst., commercial, feed grade); 1,000 mg pantothenic acid (Ca d-pantothenate); 60,000 mg choline (chloride); 5,000 mg iron (iron carbonate); 5,000 mg zinc (zinc sulfate); 6,000 mg manganese (manganous oxide); 1,000 mg copper (copper oxide); 45 mg iodine (calcium-iodate); 20 mg selenium (sodium-selenite); 140 g sodium (NaCl); 55 g magnesium (magnesium sulfate); carrier: calcium carbonate (calcium min 38%); Quantum Blue 5G: 41,666 FTU/g; Econase XT 5P:133,333 BXU/g; Monteban G100: 5,833 mg; 5P:133'3333 BXU.

[3]Nitrogen-corrected apparent metabolizable energy estimated from chemical composition of feed ingredients (based on the EU Regulation—Directive 86/174/EEC): 0.1551 × % crude protein + 0.3431 × % ether extract + 0.1669 × % starch + 0.1301 × % total sugar.

[4]BXU: beta-xylanase unit.

[5]FTU: phytase unit.

[6]LSU(F): muramidase unit.

temperature. Fixed tissues were dehydrated in graded series of ethanol, embedded in paraffin, cut to 5 $\mu$m thin sections, put on slides, de-waxed in xylene, rehydrated, and stained accordingly to H&E or AB/PAS standard staining protocols. For each sample, 3 to 6 slides were prepared and examined under a light microscope (Nikon, Tokyo, Japan). The digitalized live pictures were transferred to a computer monitor and analyzed employing the computer-assisted image analysis program NIS-Elements AR (Nikon Instruments INC.). Villus length and crypt depth were measured on the H&E samples with ×50 magnification. Only parts of the sections were measured, where villi were cut completely from top to bottom and the most crypts were also cut vertically. Villus length was defined as the distance from the tip of the villus to the villus–crypt junction. The crypt depth was determined as the distance from the villus–crypt junction to the crypt´s base. In total, 20 villi and crypts were measured per sample and the mean values were taken for further calculations. The ratio of villus length to crypt depth (**VLCDR**) was calculated.

The number of goblet cells was counted on the AB/PAS samples with ×600 magnification. They were detected via a color threshold with the automatic counting tool. A region of interest (**ROI**) within the field of view was defined. Fields were only measured, if the epithelium was cut tangentially on the level of the goblet cells and filled the whole ROI. Ten fields were measured per sample. For immunohistochemical detection of CD45-positive cells (leukocytes), mouse anti-chicken CD45 PE (Southern Biotech, Birmingham, UK, Cat No 8270–09, Clone LT40) was used. Mouse IgM-PE (Southern Biotech, Birmingham, UK, Cat No 0101–09) was used as the isotype control. The number of CD45-positive cells in the epithelium was measured on the samples with ×600 magnification. They were detected via a color threshold with the automatic counting tool. An ROI within the field of view was defined. Fields were only measured, if the epithelium was cut tangentially and filled the whole ROI. A total of 30 fields were measured per sample to cover all levels within the epithelium homogeneously (above, on the level, and under the nucleus). Results for goblet cells and CD45-positive

**Table 2.** Ingredients and nutritional characteristics of the grower diets from day 22 to 35 of age (as is).

| Treatment groups[1] | | Control | Low | Medium | High |
|---|---|---|---|---|---|
| Ingredients | | | | | |
| Maize | g/kg | 308.7 | 308.7 | 308.7 | 308.7 |
| Wheat | g/kg | 295.7 | 295.7 | 295.7 | 295.7 |
| Soybean meal (CP 47%) | g/kg | 293 | 293 | 293 | 293 |
| Soybean oil | g/kg | 58 | 58 | 58 | 58 |
| Limestone | g/kg | 10.3 | 9.9 | 9.8 | 9.6 |
| Monocalcium phosphate | g/kg | 13.8 | 13.8 | 13.8 | 13.8 |
| Premix[2] | g/kg | 12 | 12 | 12 | 12 |
| Titanium(IV) dioxide | g/kg | 3 | 3 | 3 | 3 |
| L-Lysine HCL | g/kg | 2.3 | 2.3 | 2.3 | 2.3 |
| DL-Methionine | g/kg | 2.3 | 2.3 | 2.3 | 2.3 |
| L-Threonine | g/kg | 0.9 | 0.9 | 0.9 | 0.9 |
| Muramidase 007 | g/kg | 0 | 0.382 | 0.534 | 0.687 |
| Analyzed nutrient composition | | | | | |
| $AME_n$[3] | MJ/kg | 12.73 | 12.73 | 12.73 | 12.73 |
| Crude protein | g/kg | 200.7 | 201.3 | 200.8 | 201.0 |
| Crude fiber | g/kg | 25.5 | 25.3 | 25.0 | 25.9 |
| Ether extract | g/kg | 80.2 | 79.7 | 78.3 | 79.0 |
| Starch | g/kg | 402.5 | 403 | 402.7 | 384.2 |
| Sugars | g/kg | 40.6 | 39.7 | 40.2 | 39.5 |
| Ca | g/kg | 8.6 | 8.6 | 8.5 | 8.5 |
| P (total) | g/kg | 7.0 | 6.8 | 6.7 | 6.8 |
| Na | g/kg | 1.6 | 1.6 | 1.7 | 1.7 |
| Xylanase | BXU[4]/kg | 16,231 | 16,188 | 16,203 | 16,172 |
| Phytase | FTU[5]/kg | 679 | 692 | 681 | 676 |
| Muramidase | LSU(F)[6]/kg | 1,850 | 26,158 | 37,605 | 41,496 |

[1]Control: control diet, low (25,000 LSU(F)/kg muramidase), medium (35,000 LSU(F)/kg muramidase), high (45,000 LSU(F)/kg muramidase).

[2]Contents per kg Premix: 600,000 I.U. Vit. A (acetate); 120,000 I.U. Vit. $D_3$; 6,000 mg Vit. E ($\alpha$-tocopherole acetate); 200 mg Vit. $K_3$ (MSB); 250 mg Vit. $B_1$ (mononitrate); 420 mg Vit. $B_2$ (cryst. riboflavin); 300 mg Vit. $B_6$ (pyridoxin-HCl); 1,500 $\mu$g Vit. $B_{12}$; 3,000 mg niacin (niacinamide); 12,500 $\mu$g biotin (commercial, feed grade); 100 mg folic acid (cryst., commercial, feed grade); 1,000 mg pantothenic acid (Ca d-pantothenate); 60,000 mg choline (chloride); 5,000 mg iron (iron carbonate); 5,000 mg zinc (zinc sulfate); 6,000 mg manganese (manganous oxide); 1,000 mg copper (copper oxide); 45 mg iodine (calcium-iodate); 20 mg selenium (sodium-selenite); 140 g sodium (NaCl); 55 g magnesium (magnesium sulfate); carrier: calcium carbonate (calcium min 38%); Quantum Blue 5G: 41,666 FTU/g; Econase XT 5P:133,333 BXU/g; Monteban G100: 5,833 mg; 5P:133'3333 BXU.

[3]Nitrogen-corrected apparent metabolizable energy estimated from chemical composition of feed ingredients (based on the EU Regulation—Directive 86/174/EEC): 0.1551 × % crude protein + 0.3431 × % ether extract + 0.1669 × % starch + 0.1301 × % total sugar.

[4]BXU: beta-xylanase unit.

[5]FTU: phytase unit.

[6]LSU(F): muramidase unit.

cells were expressed as cells per 0.1 $mm^2$ of epithelial area. The data are presented in Table 5.

## Chemical analysis

Chemical analysis of feed samples included Weende constituents and also calcium, phosphorus, and sodium. Analyses were in accordance to the methods issued by VDLUFA (2003) and were as follows, dry matter: VDLUFA III 3.1; CP: VDLUFA III 4.1.1 modified according to macro-N determination (vario Max CN); crude fiber: VDLUFA III 6.1.4; EE: VDLUFA III 5.1.1; starch: VDLUFA III 7.2.1; total sugars: VDLUFA III 7.1.1; Ca: VDLUFA VII 2.2.2.6; P: VDLUFA VII 2.2.2.6; Na: VDLUFA VII 2.2.2.6. Titanium dioxide content was measured using the method described by Short et al. (1996). The muramidase activity of feed samples was determined according to the method described by Lichtenberg et al. (2017). The ileal pooled digesta was also analyzed for EE, CP, Ca, P, and $TiO_2$ content, using the same methods mentioned above.

## Statistical Analysis

Data were subjected to ANOVA using the GLM procedure of SPSS 21.0 (SPSS Inc., Chicago, IL). Treatment means were separated by the Tukey least significant difference post hoc test at $P \leq 0.05$ statistical level. Orthogonal polynomial contrasts were used to examine linear and quadratic effects of different inclusion levels of the muramidase. The replicate pen was the experimental unit for all variables measured.

# RESULTS

Results of analyses of the muramidase activity in the diets confirmed the correct addition of the test products within the range of the expected values ± 20% (Tables 1 and 2).

In the first week of age, adding muramidase to broiler diets linearly improved BWG and FCR ($P < 0.001$ and $P = 0.012$, respectively), and birds fed the highest dosage of muramidase had better BWG and FCR

**Table 3.** Effect of different inclusion levels of muramidase on growth performance[*] of broiler chickens.

| | | | | | | *P* value | | |
|---|---|---|---|---|---|---|---|---|
| Treatment groups[1] | | Control | Low | Medium | High | | Linear | Quadratic |
| | | | | First week (day 1 to 7 of age) | | | | |
| Body weight gain | g | $100 \pm 3^{a}$ | $102 \pm 2^{a,b}$ | $102 \pm 2^{a,b}$ | $104 \pm 2.9^{b}$ | 0.003 | <0.001 | 0.853 |
| Feed intake | g | $112 \pm 6$ | $110 \pm 5$ | $110 \pm 3$ | $111 \pm 2.9$ | 0.722 | 0.655 | 0.362 |
| Feed conversion ratio[2] | | $1.117 \pm 0.059^{a}$ | $1.080 \pm 0.046^{a,b}$ | $1.082 \pm 0.032^{a,b}$ | $1.066 \pm 0.038^{b}$ | 0.049 | 0.012 | 0.419 |
| | | | | Starter period (day 1 to 21 of age) | | | | |
| Body weight gain | g | $871 \pm 20$ | $881 \pm 19$ | $884 \pm 18$ | $881 \pm 17$ | 0.350 | 0.163 | 0.249 |
| Feed intake | g | $1058 \pm 26$ | $1051 \pm 28$ | $1052 \pm 22$ | $1047 \pm 18$ | 0.727 | 0.288 | 0.922 |
| Feed conversion ratio[2] | | $1.215 \pm 0.029^{a}$ | $1.194 \pm 0.014^{a,b}$ | $1.190 \pm 0.017^{b}$ | $1.188 \pm 0.018^{b}$ | 0.009 | 0.003 | 0.116 |
| | | | | Grower period (day 22 to 35 of age) | | | | |
| Body weight gain | g | $1301 \pm 57$ | $1333 \pm 44$ | $1333 \pm 30$ | $1334 \pm 35$ | 0.156 | 0.069 | 0.211 |
| Feed intake | g | $1972 \pm 103$ | $1966 \pm 70$ | $1954 \pm 51$ | $1952 \pm 42$ | 0.882 | 0.432 | 0.916 |
| Feed conversion ratio[2] | | $1.518 \pm 0.073^{a}$ | $1.475 \pm 0.022^{a,b}$ | $1.466 \pm 0.029^{b}$ | $1.463 \pm 0.031^{b}$ | 0.013 | 0.004 | 0.119 |
| | | | | Overall period (day 1 to 35 of age) | | | | |
| Body weight gain | g | $2172 \pm 51$ | $2214 \pm 47$ | $2217 \pm 37$ | $2216 \pm 34$ | 0.060 | 0.018 | 0.087 |
| Feed intake | g | $3030 \pm 113$ | $3017 \pm 78$ | $3006 \pm 61$ | $2999 \pm 42$ | 0.775 | 0.301 | 0.900 |
| Feed conversion ratio[2] | | $1.395 \pm 0.043^{a}$ | $1.363 \pm 0.015^{b}$ | $1.356 \pm 0.023^{b}$ | $1.354 \pm 0.016^{b}$ | 0.001 | <0.001 | 0.008 |
| EPEF[3] | | $443 \pm 18.1^{b}$ | $463 \pm 10.0^{a}$ | $467 \pm 11.9^{a}$ | $467 \pm 11.1^{a}$ | <0.001 | <0.001 | 0.421 |

[1]Control: control diet, low (25,000 LSU(F)/kg muramidase), medium (35,000 LSU(F)/kg muramidase), high (45,000 LSU(F)/kg muramidase).
[2]g feed per g body weight gain.
[3] European poultry efficiency factor: averaged grams gained per day × survival rate (%) ÷ feed conversion ratio × 10.
[*]Data are means of 12 replicate pens with 20 birds per pen.
[a,b]Different superscripts within lines indicate levels of significance at $P \leq 0.05$.

**Table 4.** Effect of different inclusion levels of muramidase on apparent ileal digestibility of nutrients[*] in broiler chickens at day 35 of age.

| | | | | | | *P* value | | |
|---|---|---|---|---|---|---|---|---|
| Treatment groups[1] | | Control | Low | Medium | High | | Linear | Quadratic |
| Crude protein | % | $72.8 \pm 4.05^{b}$ | $74.7 \pm 4.17^{a,b}$ | $76.6 \pm 3.12^{a,b}$ | $77.5 \pm 2.80^{a}$ | 0.011 | 0.001 | 0.606 |
| Ether extract | % | $91.3 \pm 1.49^{b}$ | $91.5 \pm 1.49^{a,b}$ | $92.9 \pm 1.78^{a,b}$ | $93.1 \pm 1.46^{a}$ | 0.009 | 0.002 | 0.955 |
| Ca | % | $53.9 \pm 1.38$ | $53.9 \pm 1.30$ | $54.3 \pm 1.46$ | $54.8 \pm 1.53$ | 0.381 | 0.100 | 0.580 |
| P | % | $60.9 \pm 2.74$ | $62.0 \pm 2.93$ | $62.8 \pm 2.28$ | $63.7 \pm 1.89$ | 0.055 | 0.007 | 0.722 |

[1]Control: control diet, low (25,000 LSU(F)/kg muramidase), medium (35,000 LSU(F)/kg muramidase), high (45,000 LSU(F)/kg muramidase).
[*]Data are means of 12 replicate pens, 3 birds per pen.
[a,b]Different superscripts within lines indicate levels of significance at $P \leq 0.05$.

compared with C group ($P = 0.003$ and $P = 0.049$, respectively). At the end of the starter and grower periods, inclusion of muramidase to broiler diets linearly decreased FCR ($P = 0.003$ and $P = 0.004$, respectively), and birds fed M and H diets had better FCR compared with those received C diet ($P = 0.009$ and $P = 0.013$, respectively). At the end of the experiment, muramidase supplementation linearly improved BWG and FCR ($P = 0.018$ and $P < 0.001$, respectively), and birds in L, M, and H groups had lower FCR compared with those in C group ($P = 0.001$). Muramidase supplementation also showed quadratic decrease in cumulative FCR at day 35 ($P = 0.008$). The EPEF standardizes technical results with taking into account feed conversion ratio, mortality, and daily weight gain. Supplementation of broiler diets with muramidase linearly increased EPEF ($P < 0.001$) and broilers fed L, M, and H diets had better EPEF compared with those fed C diet ($P = 0.001$). Except for cumulative BWG at day 35, inclusion of different levels of muramidase into broiler diets had no impact on FI and BWG of broilers during the starter, grower, and entire experimental periods ($P > 0.05$).

Adding muramidase to broiler diets linearly improved AID of CP and EE ($P = 0.001$ and $P = 0.002$, respectively), and broilers receiving the highest dosage of muramidase had remarkably better AID of CP and EE compared with C group ($P = 0.011$ and $P = 0.009$, respectively). There were no significant differences between experimental groups for AID of Ca ($P > 0.05$), while inclusion of muramidase to broiler diets linearly increased AID of P ($P = 0.007$).

Muramidase inclusion in broiler diets linearly increased VLCDR and decreased number of CD45 cells in the ileum ($P = 0.045$ and $P = 0.003$, respectively). Inclusion of medium and high dosages of Muramidase 007 in broiler diets resulted in a reduction in number of CD45 cells in the ileum ($P \leq 0.05$). However, the number of CD45 cells in the ileum of broilers fed L diet was similar to the other 3 groups. The histological evaluations did not reveal any other considerable

**Table 5.** Effect of different inclusion levels of muramidase on morphometry and immunohistochemically analysis* in the mid-jejunum and -ileum of broiler chickens at day 35 of age.

| Treatment groups[1] | | Control | Low | Medium | High | P value | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | | Linear | Quadratic |
| Mid-jejunum | | | | | | | | |
| Villi length | $\mu$m | 1239 ± 318 | 1358 ± 437 | 1371 ± 209 | 1481 ± 154 | 0.468 | 0.131 | 0.962 |
| Crypt depth | $\mu$m | 142 ± 13 | 149 ± 34 | 157 ± 28 | 161 ± 33 | 0.558 | 0.160 | 0.883 |
| Villi length/crypt depth | | 8.7 ± 2.35 | 9.6 ± 3.31 | 8.9 ± 1.34 | 9.4 ± 1.36 | 0.840 | 0.744 | 0.820 |
| CD45-positive cells | n/0.1 mm$^2$ | 372 ± 66 | 362 ± 82 | 350 ± 33 | 405 ± 120 | 0.584 | 0.524 | 0.267 |
| Goblet cells | n/0.1 mm$^2$ | 605 ± 129 | 633 ± 243 | 635 ± 78 | 706 ± 91 | 0.588 | 0.212 | 0.688 |
| Mid-ileum | | | | | | | | |
| Villi length | $\mu$m | 808 ± 112 | 882 ± 149 | 877 ± 115 | 903 ± 76 | 0.400 | 0.136 | 0.561 |
| Crypt depth | $\mu$m | 117 ± 4 | 122 ± 13 | 116 ± 3 | 115 ± 5 | 0.234 | 0.242 | 0.297 |
| Villi length/crypt depth | | 6.9 ± 1.15 | 7.2 ± 0.88 | 7.6 ± 1.06 | 7.9 ± 0.88 | 0.244 | 0.045 | 0.978 |
| CD45-positive cells | n/0.1 mm$^2$ | 462 ± 54[a] | 396 ± 61[a,b] | 338 ± 78[b] | 363 ± 81[b] | 0.008 | 0.003 | 0.074 |
| Goblet cells | n/0.1 mm$^2$ | 1020 ± 78 | 984 ± 144 | 957 ± 83 | 946 ± 97 | 0.501 | 0.141 | 0.730 |

[1]Control: control diet, low (25,000 LSU(F)/kg muramidase), medium (35,000 LSU(F)/kg muramidase), high (45,000 LSU(F)/kg muramidase).
*Data are means of 8 replicate-pens, 1 bird per pen.
[a,b]Different superscripts within lines indicate levels of significance at $P \leq 0.05$.

impact either on the morphometric values or on the quantity of the lymphocytes (CD45) or goblet cells ($P > 0.05$).

## DISCUSSION

In the present study, inclusion of Muramidase 007 in broiler diets linearly increased BWG and decreased FCR at the early life stage of chickens ($P \leq 0.05$). However, at the end of the experiment, inclusion of the muramidase could linearly improve the cumulative FCR, BWG, and EPEF ($P \leq 0.05$). The results are in agreement with data from Lichtenberg et al. (2017), where inclusion of 45,000 LSU(F)/kg (686 ppm) of microbial muramidase (Muramidase 007) to the diets of broilers improved FCR at the end of the study (day 42). Similarly, other studies on the use of muramidase from different origins, e.g., modified rice expressing lysozyme (Humphrey et al., 2002) or HEW lysozyme (Abdel-Latif et al., 2017), in poultry diets have also reported improvement in feed efficiency. In contrast, adding 100 ppm HEW lysozyme preparation (4800 units/mg enzymatic activity) to broiler diets had no effects on BWG and FCR at different life stages of healthy chickens (Gong et al., 2017). In *C. perfringens* challenge studies showed dietary HEW lysozyme improved growth performance of broiler chickens as compared with challenged birds in control group (not provided with lysozyme), but not compared with unchallenged birds (Liu et al., 2010; Zhang et al., 2010).

The histomorphological evaluation of jejunum in the present study showed no impact on the morphometric variables as well as the quantity of the lymphocytes (CD45) or goblet cells. However, in the ileum, the muramidase supplementation linearly increased VLCDR and decreased number of CD45 cells ($P \leq 0.05$). Data regarding the effect of microbial muramidase on intestinal histomorphology of broilers and pigs are limited, and reported results on non-microbial muramidase have been inconsistent. In a study conducted by Humphrey et al. (2002), chickens fed a conventional diet containing 10% modified rice expressing lysozyme showed longer villi in the duodenum but not in the ileum, while the number of leukocytes in their ileal lamina propria decreased. However, inclusion of HEW lysozyme in broiler diets increased villi length and crypts depth in the jejunum (Abdel-Latif et al., 2017). Supplementation of weaning piglet diets with 90 mg/kg HEW lysozyme increased VLCDR in the duodenum and jejunum, while it had no effect on villus length and crypt depth in the duodenum, jejunum, and ileum (Long et al., 2016). Supplementation of milk replacer (for weaning piglets) with liquid HEW lysozyme (100 mg/L) or antibiotic, similarly, deepened crypt depth in the jejunum and ileum, and also increased villus length in the ileum (May et al., 2012). Weaned pigs that received diets containing a water-soluble HEW lysozyme showed longer villus but similar crypt depth and VLCDR in the ileum compared with those received control diet with no additive (Nyachoti et al., 2012).

A well-functioning and healthy gut is the cornerstone for the optimum performances of poultry. When the gut function and health are impaired, digestion and absorption of nutrients are affected and thus the growth performance might be compromised. AID measurement is a quantitative estimation of nutritional and physiological phenomena related to digestion capacity and GI functionality. In the current study, supplementation of broiler diets with Muramidase 007 linearly improved AID of CP, EE, and P ($P < 0.05$) which might suggest an improvement in the digestion capacity and GI functionality. Even though a phytase was included in all the diets, muramidase groups showed a linear improvement in AID of P, which might indicate that muramidase could synergistically enhance phytase activity. To our best knowledge, there is no data available evaluating the impact of muramidase (microbial and non-microbial) inclusion in broiler diets on AID of nutrients. However, just recently Ma et al. (2017) studied the inclusion of 1 g/kg HEW lysozyme into

barrows diets and showed no effect on apparent total tract digestibility of gross energy, DM, and CP. It is noteworthy that inclusion of HEW lysozyme in pig and poultry diets has shown beneficial impacts on the gut health, integrity, microbiota, and antioxidant status as well as intestinal barrier and nonspecific immunity (Liu et al., 2010; Oliver and Wells, 2015; Long et al., 2016; Abdel-Latif et al., 2017; Gong et al., 2017; Lichtenberg et al., 2017; Ma et al., 2017), which could reportedly lead to better digestion and absorption capacity.

The turnover of the PGNs is a very common pathway for cell wall recycling of bacteria (Mayer, 2012). Gram-positive bacteria break down and efficiently recycle approximately 60% of the mature PGNs of their side wall during cell elongation and approximately 30% of newly deposited septal PGNs during cell division. During the growth and maturation, the PGN polymer is degraded by different PGN hydrolases, such as muramidase, glucosamidase, amidase, and peptidases (produced by the host and neighboring microbiota). As a result, muropeptides (breakdown products of bacteria PGNs) are shed from the cell wall into the gut (Traub et al., 2006; Wheeler et al., 2014). It has been reported that muropeptides modulate inflammatory response in the GI tract (Traub et al., 2006). Thus, it can be speculated that the hydroxylation of the PGNs by the dietary Muramidase 007 and production of muropeptides could possibly contribute to a modulation of the inflammatory response in the GI tract, and thus explain the benefits observed on the GI health and functionality. Furthermore, it should be taken into account that, in a normal conventional GI tract environment, where symbionts are dominant, PGNs can be accumulated and a PGN-rich condition can be created (Lee and Hase, 2014). The consequence of PGNs accumulation on the intestinal lumen remains to be investigated in detail, but it could be speculated that accumulation of bacterial cell wall fragments on the surface of the gut could impair nutrient digestion and absorption and the dietary muramidase in the present study might have counteracted this impact.

In conclusion, inclusion of the current microbial muramidase in broiler diets could improve the digestion capacity, and subsequently improve growth performance. Thus, microbial muramidase can be used as an efficient feed additive in broiler nutrition, although the biological mechanisms leading to these beneficial impacts deserve to be studied further.

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