Dietary microbial muramidase improves feed efficiency, energy and nutrient availability, and welfare of broilers fed commercial type diets containing exogenous enzymes

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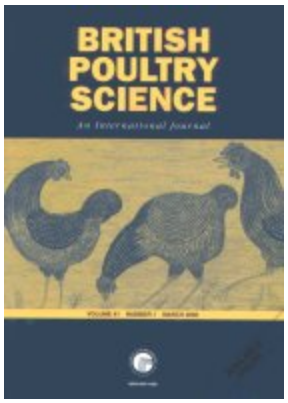

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Dietary microbial muramidase improves feed efficiency, energy and nutrient availability, and welfare of broilers fed commercial type diets containing exogenous enzymes

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Abstract

1. The aim of this study was to evaluate the effect of graded levels of the microbially-derived feed lysozyme, muramidase (MUR) on feed intake (FI), weight gain (WG), feed conversion ratio (FCR), European Performance Index (EPI), dietary N-corrected apparent metabolisable energy (AMEn), footpad dermatitis score (FPD) and other welfare variables, when fed to broilers from 0 to 42d age.

2. A four-phase dietary program and four experimental pelleted diets were used; a control diet (following breeder recommendations without MUR supplementation), and three diets based on the control diet supplemented with 25,000, 35,000 and 45,000 LSU (F)/kg of MUR, respectively. In addition, all experimental diets contained exogenous xylanase, phytase and a coccidiostat. Each diet was fed to birds in 24 pens (20 male Ross 308 chicks in each pen) following randomisation. Dietary AMEn was determined at 21 d of age, and FPD was evaluated at the end of the study. Data were analysed by ANOVA, using orthogonal polynomials for assessing linear and quadratic responses to MUR activity.

3. The inclusion of MUR did not change FI ($P>0.05$), but increased WG in a linear manner ($P<0.05$) and reduced FCR in a quadratic manner, with optimum WG and FCR observed in birds fed approximately 35000 LSU (F)/kg. In accordance with the improvement in FCR, 35000 LSU (F)/kg MUR supplementation produced the highest EPI ($P<0.05$). FPD score was

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linearly decreased with increased addition of MUR ($P<0.05$). Dietary AMEn responded in a quadratic fashion to the MUR inclusion, as the highest values were obtained with the highest inclusion rate ($P<0.05$).

4. In conclusion, the results showed that inclusion of MUR improved feed efficiency and the foot health of birds.

Key words: Muramidase, feed efficiency, metabolisable energy, footpad dermatitis.

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Introduction

The use of feed additives to improve the efficiency of growth and/or egg production, prevent disease and improve feed utilisation is a common strategy to improve efficiency in the poultry industry (Pirgozliev *et al.,* 2019). Exogenous enzymes are the most commonly used feed additives. The enzymes widely used by the industry are non-starch polysaccharidases that cleave the non-starch polysaccharides in viscous cereals and microbial phytases that target the phytate-complexes in plant ingredients (Pirgozliev *et al.,* 2010; Adeola and Cowieson, 2011; Ravindran, 2013). Recently a new category of feed enzymes, microbial muramidase (MUR) have become available, in which the substrate is not present in the feed but already present in the gastrointestinal tract. Muramidases (EC 3.2.1.17), also known as lysozyme or N-acetylmuramidase, are naturally found in a great variety of animal secretions, plants, or micro-organisms. Muramidases are glycosyl hydrolytic enzymes that cleave the β-1, 4 glycosidic linkages between N-acetylmuramic acid and N-acetyl glucosamine in the carbohydrate backbone of bacterial cell wall components, called peptidoglycans (PGNs). Recent studies have demonstrated the efficacy of microbial MUR on feed efficiency and gastrointestinal tract functions, enhancing nutrient digestibility and absorption (Goodarzi Booronjeri *et al.,* 2019; Sais *et al.,* 2019). Lichtenberg *et al.* (2017) suggested that catalysing the depolymerisation of PGNs from the bacterial cell debris present in the gut, as a result of the continuous bacterial turnover, may best describe the mode of action of this enzyme. During this process, 50% of the pre-existing PGNs in a bacterial cell are released from the wall and recycled within one generation (Reith and Mayer, 2011), although the fate of the remaining 50% is unclear. It can be speculated that accumulation of bacterial cell wall fragments at the gut surface could impair nutrient digestion and absorption and, in that case, the inclusion of microbial MUR in broiler diets could result in better nutrient availability and higher growth performance (Goodarzi Boroojeni *et al.,* 2019). Thus, the combined application of different categories of enzymes in

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commercial poultry diets may result in additive or synergistic effects on nutrient utilisation and animal performance.

The present study investigated the impact of different inclusion levels of microbial MUR on growth performance, including feed intake (FI), weigh gain (WG) and feed conversion ratio (FCR), dietary N-corrected apparent metabolisable energy (AMEn), dry matter (DMR), organic matter (OMR), nitrogen (NR) and fat retention (FR) coefficients, sialic acid (SA) in excreta, foot bad dermatitis score (FPD), European poultry efficiency factor (EPEF) and some litter quality variables when fed to broilers from 0 to 42d age.

Materials and methods

The experiment was conducted at the National Institute of Poultry Husbandry (NIPH) and approved by the Research Ethics Committee of Harper Adams University, Newport, UK.

Animals and experimental design

A total of 1960, male, Ross 308 broilers were obtained from a commercial hatchery (Cyril Bason Ltd, Craven Arms, UK). On the arrival, 1920 birds were divided into 96 floor pens with 20 birds in each (excluding ill and malformed birds). Each of the 96 pens had a solid floor and measured 2.1 m^2 and bedded with wood shavings.

The room temperature was approximately 32ºC at day old and was gradually reduced to about 20ºC at 21 days of age. A standard lighting program for broilers was used, decreasing the light:dark ratio from 23h:1h from one day old to 18h:6h at seven days old, which was maintained until the end of the study. Access to feed and the water was *ad libitum*.

Four starter (day 1 to 10), grower (day 11 to 20), finisher-1 (day 21 to 35) and finisher-2 (day 35 to 42) wheat-soybean diets were produced (control; C) , three containing different levels of

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microbial MUR (Balancius™, DSM Nutritional Products Ltd, Kaiseraugst, Switzerland); low (L, 417 g/t; 25,000 LSU(F)/kg, medium (M, 583 g/t; 35,000 LSU(F)/kg), and high (H, 750 g/t; 45,000 LSU(F)/kg). Each single unit of LSU(F) is defined as the amount of enzyme that increases the fluorescence of 12.5 µg/ml fluorescein-labelled 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 I.

The tested MUR product was included in powder form with a minimum analysed MUR activity of 60,000 LSU(F)/g product. Diets were supplemented with exogenous xylanase (RONOZYME®WX, endo-1,4-beta-xylanase; DSM Nutritional Products Ltd, Kaiseraugst, Switzerland), phytase (RONOZYME® HiPhos; DSM Nutritional Products Ltd, Kaiseraugst, Switzerland) and coccidiostat (CLINACOX®, Elanco Ltd., Guelph, CA). No antibiotic was included in feed during the experimental period. The diets were isocaloric and isonitrogenous for each feeding phase, and met or exceeded breeder recommendations (Aviagen Ltd, Edinburgh, UK). The composition of the experimental diets is shown in Table 1.

Table 1 here

Mortality was recorded daily. A visual assessment for litter score of the entire pen was performed at 34 d old, using a five point scoring system, from 1 to 5, as previously described (Da Costa *et al.,* 2014; Mirza *et al.,* 2016). A lower score indicated better litter quality. The litter pH was determined at 35 d of age using a pH probe with a stainless steel penetration blade directly into the litter in four different sides in each pen. The pH probe was attached to a Hanna HI 99163 meter (Hanna Instruments Ltd, Bedfordshire, UK). Litter dry matter was determined at 35 d of age by taking five samples from the same locations of the floor in each pen, including

the area near the drinker, and drying them in an oven (see method below). The samples were then homogenised, milled and stored dry before further analysis.

Footpad and hock lesions were assessed and given a score at 35 d of age for both the left and right leg of all birds, and classified according to a scale published by Hocking *et al.,* (2008) from 0 (no lesion) to 4 (very severe lesions). A mean value per pen for each of the measurements was used in statistical analysis.

At 17 d of age, two randomly selected birds from each pen were transferred to one of 96 raised-floor battery pens (60 × 60 cm floor area) in a controlled environment room. Each pen was equipped with a metal feeder, providing 40 cm feeding space, and two nipple drinkers with spill cups. Treatments were randomly allocated to the pens. Feed and water were offered for *ad libitum* consumption. The selected birds were kept in the pens for 72 h, and total excreta were collected three times (every 24 h) from the trays beneath, and spilled feed and feathers were removed before weighing. Feed intake was weighed for the same period. The N-corrected apparent metabolisable energy (AMEn) of diets was determined following the procedure of Hill and Anderson (1958).

The coefficients of apparent retention of dietary dry matter (DMR) and N (NR) retention coefficients were determined as the difference between nutrient intake (feed intake multiplied by the nutrient content in feed) and nutrient output (excreta voided for 72 h multiplied by the nutrient content in excreta) divided by the nutrient intake.

The European Poultry Efficiency Factor, which standardises technical results by considering FCR, mortality and daily weight gain, was determined for the broilers from 0 to 42 d age.

Chemical analysis

Dry matter in litter, feed and excreta was determined by drying samples in a forced draft oven at 105°C to a constant weight (AOAC 2000; method 934.01). Crude protein (6.25 × N) in litter,

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feed and excreta samples was determined by the combustion method (AOAC 2000; method 990.03) using a LECO FP-528 N (Leco Corp., St. Joseph, MI, USA). Oil (as ether extract) was analysed using diethyl ether by the ether extraction method (AOAC 2000; method 945.16) using a Soxtec system (Foss Ltd., Warrington, UK). The gross energy (GE) values for feed and excreta samples were determined in a bomb calorimeter (model 6200; Parr Instrument Co., Moline, IL, USA), with benzoic acid used as the standard.

Endogenous mucin in the dry excreta was measured using the concentration of the SA as a marker, following the periodate-resorcinol method (Jourdian *et al.,* 1971). In brief, the method involves conversion of free and glycosidically bound SA to chromogenic substances, by treatment with periodic acid followed by resorcinol. The colour of the samples was stabilised by 2-methyl-propan-2-ol, and, after centrifugation, the absorbance of the supernatant was determined spectrophotometrically at 630 nm (Spectronic 301; Milton Roy Company, Warminster, PA). This procedure detected total, free, and glycosidically bound N acetyl neuraminic (sialic) acid. The MUR activity in the feed samples was determined according to the method described by Lichtenberg *et al.* (2017).

Statistical analysis

Prior to statistical analyses, data were checked for normality and homogeneity, and transformations were deemed not necessary. Statistical analyses were performed using GenStat (18th edition) statistical software package for Windows (IACR, Rothamstead, Hertfordshire, UK). The comparison between the experimental results was performed by ANOVA, using orthogonal polynomials for testing linear and quadratic responses to MUR inclusion. Differences were reported as significant at $P<0.05$, and trends towards significance ($P<0.1$), were included in the report.

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Results

The birds remained healthy throughout the study period. No adverse effects due to feeding the experimental diets were observed, and the overall mortality was low at 3.4% and not treatment related. The determined chemical composition of the diets is presented in Table 1 and agreed with the calculated values.

Table 1 here

Results of analyses of MUR activity in the diets confirmed the correct addition of the product within the range of the expected values ± 20% (Table 2).

Table 2 here

During the first three weeks of the feeding trial there were no effects ($P>0.05$) of diet on any growth performance variables, although birds fed the control diet tended ($P=0.053$) to have the lowest WG during the starter phase (1-10 d; Table 3).

A change in performance was observed at 35 d of age when weight gain of the birds was improved in a significant linear fashion ($P<0.05$) with increasing MUR dosage. The high dosage of MUR gave the lowest FCR, although the response was curvilinear ($P<0.05$), i.e. low MUR dosage produced a higher FCR compared to medium and high dosages. Overall, for the entire period from one to 42 d of age, weight gain increased in a dose dependent linear manner ($P<0.001$). The significant quadratic response of FCR at 42 d to MUR supplementation

(P=0.010) suggested that the optimum inclusion level at this age was at 35,000 LSU(F)/kg, where FCR was 2.6% lower than the control. In agreement with the FCR at 42 d of age, the EPEF responded in the same way to MUR activity (P=0.016), being 6.7% higher than the control when the diet was supplemented with 35,000 LSU (F)/kg. The liveability of the birds was unaffected (P>0.05) by MUR dosage.

The footpad dermatitis score, determined at 35 d of age, was reduced in a dose dependent linear manner (P<0.001; Table 3) in agreement with the improved WG and FCR for the same period.

Table 3 here

Dietary MUR significantly alter the litter dry matter, pH, N content or footpad dermatitis score (P>0.05; Table 4). Fat retention increased in a dose dependent linear manner (P<0.001; Table 4).

Table 4 here

There were no differences (P>0.05) in SA excretions. Exogenous MUR supplementation significantly improved (P<0.05) dietary AMEn, and the coefficients of retention of dry matter, organic matter and nitrogen (Table 5) in a quadratic manner.

Table 5 here

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Discussion

The positive responses in the growth performance variables and EPEF in this study are in accordance with recently published studies. When feeding the same levels of the same MUR product, Goodarzi Boroojeni *et al.* (2019) found a linear increase in WG and decrease in FCR at 35 d of age and the supplementation improved EPEF in similar way as in the present paper. Sais *et al.,* (2019) reported reduced FCR in broilers fed MUR from day old to 36 d age. Most importantly, the improvement in FCR at 42 d of age in the current study agreed with the findings of Lichtenberg *et al.* (2017), who fed the same dosage of the same enzyme to broilers. The latter authors found an even greater improvement in final weight of birds, although they were fed much higher MUR dosages (225,000 and 450,000 LSU (F)/kg), although no changes at FCR were noted.

Studies on the use of MUR from different origins, e.g. modified rice expressing lysozyme (Humphrey *et al.,* 2002) or hen egg-white (HEW) lysozyme (Abdel-Latif *et al.,* 2017), in broiler diets have been reported to improve feed efficiency. However, Gong *et al.* (2017) found no effect on growth performance, but saw changes in the microbiome when feeding a HEW lysozyme preparation to broilers. Liu *et al.* (2010) and Zhang *et al.* (2010), reported improved growth when HEW lysozyme was fed to *Clostridium perfringens* challenged birds, but not in the unchallenged control group.

The variation in growth responses between published reports may be attributable to differences in dietary formulations, enzyme dose, application or the origin of the lysozyme or the simultaneous use of other enzymes. Given the diversity in origin between different lysozymes evaluated *in vivo*, it can be speculated that the mode of action can differ. In the current study, the microbial-derived product used was encoded by the *MUR* gene from the fungus *Acremonium alcalophilum* and was assessed to ensure it did not to possess any antibacterial activities at the intended doses (EFSA, 2018). Lichtenberg *et al.* (2017) showed an increase in

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feed efficiency, without any significant differences in the caecal microbiome for microbially-derived MUR supplemented broiler diets.

In the current study, significant growth performance in response to dietary MUR was only observed in birds after 21 d of age. This suggested that the beneficial effect of MUR was related to the changing importance of the caeca in birds as they aged, as at 7 d of age the caeca represents only 13% of the weight of the small intestine, whereas at 35 d it comprises 24% of the small intestine (Yang *et al.,* 2020). Apajalathi *et al.* (2002) reported that the numbers of microbes reach 10^{11}/g of caecal digesta and 10^{9}/g of ileal digesta during the first three days post hatch, and remain relatively stable for the following 34 d. As feed intake and the absolute size of the gastrointestinal tract (GIT) increases with the age of the birds, it is logical to assume that the content of digesta, i.e. the total number of microbes in the GIT, increases proportionally. The life span of bacteria is relatively short (Fuller, 1978) and a continuous and natural bacterial turnover occurs, releasing bacterial cell debris into the GIT. Through this process, in one generation, up to half of the pre-existing PGNs from the bacterial cell wall is released and recovered (Reith and Mayer, 2011). However, it is still unclear what happens with the remaining PGNs, and, as birds age, their GIT may accumulate bacterial cell debris, including PGNs. This might explain why the improvement of growth performance was only seen in older birds in the present study.

Metabolisable energy is a measurement of the available energy from dietary carbohydrates, fats and proteins, hence, it was expected that an improvement in nutrient retention coefficients would improve dietary AMEn (Woods *et al.,* 2020). The main ingredient in the diets was wheat, which may cause an increase in digesta viscosity due to high non-starch polysaccharide (NSP) content, that can reduce energy and nutrient availability (Pirgozliev *et al.,* 2015). Although viscosity was not measured in the reported study, the quadratic response between AMEn and the majority of the nutrients suggested that MUR may have an impact on digesta viscosity.

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However, further research into any interaction between MUR and other feed additives is warranted. Zanella *et al.* (1999) found that metabolisable energy and nutrient digestibility differed when determined using ileal digesta or excreta. This may provide an alternative explanation to the quadratic responses seen to MUR in the current study, where AMEn was performed on excreta and was linear, whereas the Goodarzi Boroojeni *et al.,* (2019) study used digesta samples for evaluation.

In addition, increased digesta viscosity has been shown to reduce conjugated bile acids, affecting fat emulsification and digestibility (Langhout *et al.,* 1997). In the present study, fat retention increased with MUR in a dose dependent linear manner. Sais *et al.* (2019) showed that MUR inclusion increased ileal apparent digestibility of fat and increased fat-soluble vitamin A in plasma at 9 d of age. This suggested that MUR improves fat digestion and absorption in young birds. Goodarzi Boroojeni *et al.* (2019) observed that supplementing MUR in a 30% wheat-based diet containing exogenous carbohydrase showed improvement in the apparent ileal digestibility of fat in a linear fashion after 35 d of supplementation. Goodarzi Boroojeni *et al.* (2019) suggested that MUR might catalyse the depolymerisation of peptidoglycans from bacterial cell debris and reduce its accumulation in the gut, thus improving nutrient utilisation. During this process, negatively charged peptidoglycans (Marquis and Bender, 1990) may lose their charge, reducing the number of interactions with fat micelles, thus benefiting fat absorption.

Sialic acid has been used as a marker to measure the dynamics of mucin secretions in excreta in enzyme fed birds. Early work with phytase (Cowieson *et al.,* 2004; Pirgozliev *et al.,* 2011) showed a reduction in SA secretion due to supplementation, although feeding an enzyme mixture to broilers (Abdulla *et al.,* 2016, 2017) did not change the concentration of SA secreted. In the current study, the SA data measured in excreta after 17 d of supplementation did not indicate differences due to MUR supplementation. Goodarzi Boroojeni *et al.* (2019) did not

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observe any significant differences in goblet cell numbers at the jejunal and ileal level after 35 d supplementation with microbial MUR in a diet containing other enzymes (phytase and xylanase). However, Sais *et al.* (2019) detected an increase in goblet cell numbers after 36 d of microbial MUR supplementation in a diet without other feed enzymes. This can probably be explained by direct or indirect changes promoted by MUR in the intestinal ecosystem or in the release of bioactive factors. The variability in response may be due to the sampling region (small intestine or excreta), maturity of the birds, method of analyses or type of diet (with or without additives), and further research is needed to explore the mode of action of this microbial MUR and its role in improving gastrointestinal function.

Improvements in litter quality and footpad dermatitis contribute to welfare in poultry. The current study showed an improvement in FPD when animals were supplemented with microbial MUR, but there was no impact on litter moisture and NH_3 concentration. An increase in litter moisture and NH_3 are the main predisposing factors for footpad dermatitis in broilers (Dawkins *et al.,* 2004), although there was no obvious correlation between the improved FPD and the litter parameters. Mirza *et al.* (2016) reported that good litter scores (based on physical appearance) were not related to litter NH_3 or pH, showing that scoring *per se* is of limited value in terms of lowering FPD incidences in poultry production. This suggests that dietary MUR may provide better nutrient availability and have a direct positive impact on the development of skin of the foot pad in poultry.

It can be concluded that the exogenous microbial MUR (Balancius ^{TM}) used in this study was effective in improving growth performance and welfare in broilers. This was attributed to improved dietary nutrient and energy availability. There is a need to study potential interactions of MUR in combination with other exogenous enzymes, plant extracts and feed additives. Strategies to incorporate MUR with other feed ingredients in poultry diets, in order to improve production and welfare, may increase the profitability of broiler production.

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Table 1. Composition and nutritive values of the experimental diets

Ingredients (g/kg)	Starter	Grower	Finisher 1	Finisher 2
Wheat	586.9	680.7	700.9	724.9
Soybean meal (CP 480)	342.7	247.1	228.3	205.8
Soybean oil	36.3	41.9	42.4	43.2
Limestone	12.8	11.3	10.7	9.9
Monocalcium phosphate	9.2	7.5	6.6	5.6
Lysine HCL	2.7	3.3	3.1	2.9
Methionine DL	3.4	3.0	2.8	2.5
L-Threonine	1.3	1.5	1.4	1.3
Salt	1.9	1.6	1.7	1.7
Sodium bicarbonate	2.5	1.8	1.7	1.7
Xylanase[1]	0.0075	0.0075	0.0075	0.0075
Phytase[2]	0.0100	0.0100	0.0100	0.0100
Premix (VitMin)[3]	0.2000	0.2000	0.2000	0.2000
Calculated values				
ME (MJ/kg)	12.70	13.20	13.29	13.40
Crude protein (g/kg)	235	198	190	181
Ether extract (g/kg)	51	57	57	58
Ash (g/kg)	53	45	43	40
Digestible Lys (g/kg)	12.9	11.0	10.4	9.7
Digestible Met+Cys (g/kg)	9.5	8.4	8.0	7.6
Ca (g/kg)	10.0	9.0	8.5	8.0
Available P (g/kg)	5.0	4.5	4.3	4.0
Determined values				
DM (g/kg)	904	902	898	898
GE (MJ/kg)	16.59	16.94	17.02	16.95
Crude protein (g/kg)	245	198	200	174
Ether extract (g/kg)	50	58	56	56
Ash (g/kg)	54	53	47	43
Xylanase (FXU/kg)	183	185	177	158
Phytase (FYT/kg)	2427	2720	2408	2537

[1] Ronozyme® WX2000: minimum 2 000 FXU/ g endo-1,4-beta-xylanase; 1 xylanase unit (FXU) is defined as the amount of enzyme that releases 7.8 µmol of reducing sugar (xylose equivalents) from azo-wheat arabinoxylan per minute at pH 6.0 and 50 C

[2] Ronozyme ® HiPhos 20000GT: minimum 20 000 FYT/ g ; 1 phytase unit (FYT) is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute under reaction conditions with a phytate concentration of 5.0 mM and pH 5.5 and temperature 37°C.

[3]The vitamin and mineral premix contained vitamins and trace elements to meet breeder's recommendation (Aviagen Ltd., Edinburgh, UK). The premix provided is as follows (units/kg diet): retinol 3600 µg, cholecalciferol 125 µg, α- tocopherol 34 mg, menadione 3 mg, thiamine 2 mg, riboflavin 7 mg, pyridoxine 5 mg, cobalamin 15 µg, nicotinic acid 50 mg, pantothenic acid 15 mg, folic acid 1 mg, biotin 200 µg, iron 80 mg, copper 10 mg, manganese 100 mg, cobalt 0.5 mg, zinc 80 mg, iodine 1 mg, selenium 0.2 mg and molybdenum 0.5 mg.

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Table 2. Analysed muramidase activity in samples of the experimental diets

		Measured activity (LSU(F)/kg			
Treatment	Inclusion level (LSU(F)/kg)*	Starter	Grower	Finisher 1	Finisher 2
Control	0	-	-	-	-
Low	25 000	26472	26469	30186	26500
Medium	35 000	31422	39569	38106	45180
High	45 000	33932	49049	53036	51650

* One unit of muramidase (LSU(F)) is the amount of enzyme that increases the fluorescence of a 12.5 µg/ml fluorescein-labelled peptidoglycan suspension by a value that corresponds to the fluorescence of 0.077 mM fluorescein isothiocyanate (FITC), per minute at pH 7.5 and 30°C.

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

						Probability		
Treatment groups[1]	Control	Low	Medium	High	SEM	P	L	Q
	Starter period (1 to 10 d old)							
Feed intake (g/b)	294	293	296	295	2.2	0.800	0.610	0.993
Weight gain (g/b)	216	222	223	223	2.5	0.173	0.053	0.277
Feed conversion ratio[2]	1.376	1.325	1.329	1.325	0.0248	0.394	0.183	0.349
	Grower period (10 to 21 d old)							
Feed intake (g/b)	1142	1134	1136	1139	7.9	0.885	0.850	0.463
Weight gain (g/b)	933	956	937	935	14.4	0.663	0.809	0.402
Feed conversion ratio	1.218	1.181	1.224	1.233	0.0317	0.670	0.538	0.469
	Finisher period 1 (21 to 42 d old)							
Feed intake (g/b)	2961	2972	2951	2978	16.5	0.682	0.698	0.632
Weight gain (g/b)	1957[a]	2008[b]	2003[b]	2014[b]	12.4	0.007	0.004	0.108
Feed conversion ratio	1.492[a]	1.454[b]	1.457[b]	1.451[b]	0.0067	<0.001	<0.001	0.019
	Overall period (1 to 42 d old)							

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Feed intake (g/b)	4565	4536	4539	4562	26.3	0.809	0.970	0.331
Weight gain (g/b)	2834[a]	2874[ab]	2910[b]	2896[b]	19.1	0.038	0.012	0.164
Feed conversion ratio	1.579[a]	1.551[b]	1.538[b]	1.547[b]	0.0069	<0.001	<0.001	0.010
Liveability (%)	96.25	96.67	97.50	96.04	0.797	0.582	0.954	0.243
EPEF[3]	403.0[a]	417.9[ab]	430.9[b]	419.1[ab]	5.43	0.007	0.014	0.016

[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] Gram feed intake per gram weight gain. [3]European poultry efficiency factor: averaged grams gained per day × survival rate (%) ÷ feed conversion ratio × 10. Data are means of 24 replicate pens with 20 birds per pen. P value describes significance between treatments determined by ANOVA. Linear (L) and quadratic (Q) effects of dietary treatment. Results are statistically significant when P≤0.05.

Table 4. Effect of dietary treatment on dry matter (DM), pH and N of litter, litter and footpad scores at 35 days of age

						Probability		
Treatment groups[1]	Control	Low	Medium	High	SEM	P	L	Q
Dry matter litter (g/kg)	0.667	0.652	0.643	0.654	0.0179	0.804	0.539	0.452
pH litter	7.35	7.44	7.53	7.46	0.120	0.770	0.451	0.510
N litter (g/kg)	40.8	41.8	40.5	40.9	0.81	0.693	0.808	0.680
Litter score	3.27	3.14	3.20	3.12	0.065	0.332	0.175	0.726
Footpad score	26.0	24.0	13.0	15.8	4.45	0.123	0.039	0.590

[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 24 replicate pens with 20 birds per pen. P value describes significance between treatments determined by ANOVA. Linear (L) and quadratic (Q) effects of dietary treatment. Results are statistically significant when P≤0.05.

Table 5. Effect of dietary treatment on N-corrected apparent metabolisable energy (AMEn), dry matter (DMR), organic matter (OMR), N (NR), fat (FR) retention coefficients and sialic acid (SA) excretions.

						Probability		
Treatment groups[1]	Control	Low	Medium	High	SEM	P	L	Q

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AMEn (MJ/kg DM)	12.98	12.62	12.96	13.26	0.162	0.062	0.985	0.007
DMR	0.705	0.681	0.696	0.717	0.0104	0.107	0.701	0.016
OMR	0.729	0.706	0.725	0.743	0.0095	0.058	0.844	0.007
NR	0.592	0.571	0.577	0.613	0.0140	0.175	0.889	0.045
FR	0.815	0.811	0.834	0.843	0.0104	0.099	0.050	0.187
SA (µg/g)	1.88	2.00	1.91	1.96	0.074	0.423	0.408	0.422
SA total (µg/24h)	32.8	37.1	31.8	34.0	0.22	0.108	0.871	0.225

[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 24 replicate pens with 2 birds per pen. P value describes significance between treatments determined by ANOVA. Linear (L) and quadratic (Q) effects of dietary treatment. Results are statistically significant when $P<0.05$.

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