The physiological response of broiler chickens to the dietary supplementation of the bacteriocin nisin and ionophore coccidiostats

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ABSTRACT The aim of this study was to investigate the effect of dietary supplementation with nisin alone or in combination with salinomycin or monensin on broiler chickens in terms of growth performance, selected blood parameters, digestive enzyme activity, apparent nutrient digestibility, and tibiotarsus mineralization, as well as selected gastrointestinal tract (GIT) organ weights, intestinal length, and central immune organ weights. Two independent experiments, each including 400 one-day-old female Ross 308 chicks differing in ionophore coccidiostats, i.e., salinomycin and monensin supplementation, were conducted. The following treatments were applied: experiment 1: NA—no additives, SAL—salinomycin (60 mg/kg diet), NIS—nisin (2,700 IU/kg diet), SAL+NIS—salinomycin (60 mg/kg diet) and nisin (2,700 IU/kg diet); experiment 2: NA—no additives, MON—monensin (100 mg/kg diet), NIS—nisin (2,700 IU/kg diet) and MON+NIS—monensin (100 mg/kg diet) and nisin (2,700 IU/kg diet). The addition of nisin with or without ionophores to the birds' diet improved broiler growth performance in terms of BWG and FCR (days 1 to 14) and BWG and FI (15 to 35 d; 1 to 35 d). Salinomycin showed effects similar to those of nisin influence on growth performance (1 to 35 d), while monensin supplementation resulted in lower BWG. Moreover, no additive effect between nisin and ionophores was observed. Nisin and salinomycin had no influence on the serum concentration of selected hormones and other blood biochemical parameters except glucose, which was reduced by nisin. A decrease in lipase activity was observed during nisin and salinomycin supplementation, while the apparent ileal digestibility of fat was not affected. However, the digestibility of crude protein increased with nisin administration. Additionally, the effects of nisin on decreasing the weight and length of GIT segments were observed. Supplementation with nisin and monensin was not associated with a negative impact on tibiotarsus mineralization and the immune organ index. This study suggests that nisin may be used in broiler nutrition as a growth promotor, with no negative influence on the bird's metabolism or immune status.

Key words: bacteriocin, broiler chicken, performance, digestibility, blood parameter

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INTRODUCTION

Bacteriocins are among the most examined antimicrobial substances used in human medicine, veterinary sciences, and the food industry (Ndoti–Nembe et al., 2015; Shin et al., 2015; Ceotto–Vigoder et al., 2016; Meira et al., 2016). These small peptides, which are ribosomally synthesized by up to 99% of all known bacteria strains, are characterized by activity against other closely related bacteria, i.e., across genera or the same species (Cotter et al., 2005). Bacteriocins are used to suppress or inhibit growth of potentially pathogenic bacteria such as *Staphylococcus aureus*, *Clostridium perfringens,* and *Campylobacter jejuni*, which are present in poultry flocks and products (Svetoch and Stern, 2010; Józefiak et al., 2012; Cotter et al., 2013). Nisin is the only lantibiotic commonly used in industry and has a broad spectrum of activity against Gram-positive and -negative bacteria (Pinilla and Brandelli, 2016).

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Bacteriocins' mode of action is based on cell membrane permeabilization or inhibition of membrane formation (Józefiak and Sip, 2013). Nisin is approved by the Food and Drug Administration, the World Health Organization, and the European Union for use as a food preservative (Joint et al., 1969; Food and Administration, 1988). However, there are no regulations on the use of nisin as a feed additive in livestock nutrition, including poultry, due to the lack of scientific data. In the available literature, only a few in vivo experiments on nisin dietary supplementation have examined its influence on the gastrointestinal tract (**GIT**) microbiota of mice, rats, rabbits, ruminants, and chickens (Bernbom et al., 2006; Santoso et al., 2006; Van Staden et al., 2011; Józefiak and Sip, 2013; Lauková et al., 2014). The results suggest that addition of dietary nisin to the diet or drinking water may decrease the proliferation of potentially pathogenic bacteria such as enterococci/streptococci, coliforms, *Clostridiae*, *Pseudomonads*, and staphylococci in the GIT. Moreover, Józefiak et al. (2013) reported that nisin in broiler chicken diets improved growth performance by suppressing the growth of certain microbes, i.e., Enterobacteriaceae and the Bacteroides-Prevotella cluster.

Kierończyk et al. (2016) confirmed that nisin may positively modulate the broiler chicken GIT microbiota by limiting growth of total number of bacteria, *Clostridium perfringens* and *Lactobacillus* sp./*Enterococcus*. Surprisingly, as indicated by the latest data, nisin may enhance growth by promoting the effect of salinomycin when both nutritional factors are added to broiler chicken diets (Kierończyk et al., 2016). However, the benefits of the combination of salinomycin and nisin on broiler growth has only been investigated in a three-week study. Longer-term experiments covering the whole broiler production period are needed, as are studies on other commonly used ionophores such as monensin.

There is currently no information on the physiological response to dietary nisin application in broiler chickens. Reddy et al. (2004) demonstrated that nisin may be absorbed by the epithelium and can be detected in the serum up to 6 h after administration. Hitherto, the effect of nisin on biochemical blood parameters has only been measured in rats and rabbits and has shown no negative impact. Additionally, there is no information on the concentration of hormones in the serum that are responsible for feed intake (FI) and consequently for performance. However, recent data show that nisin in chicken diets may increase FI and thus body weight gain (BWG), especially in the first period of rearing (1 to 14 d). This effect could also result from changes in the concentration of blood hormones (Kierończyk et. al., 2016). Moreover, the skeletal abnormalities associated with nutritional factors and dietary imbalances are among the most important issues in poultry production and management (Kierończyk et al., 2017). The literature contains frequent references to the positive influence of lactic acid bacteria on bone mineralization (Ziaie et al., 2011). It is well known that most of the pro- and synbiotics in use contain bacteriocinogenic species, which may affect the tibiotarsus mineral content. However, the effects of bacteriocins have not been taken into consideration.

An exploration of the physiological response of broiler chickens to dietary supplementation with nisin is crucial for future EU legislation recognizing nisin as a potential feed additive. Therefore, the aim of this study was to investigate the physiological response of broilers to nisin in combination with salinomycin and monensin, considering growth performance, selected blood parameters, digestive enzyme activity, apparent nutrient digestibility, tibiotarsus mineralization, selected GIT organ weights, intestinal length, and central immune organ weights as outcome variables.

MATERIAL AND METHODS

All procedures and experiments complied with the guidelines of and were approved by the Local Ethics Commission of the Poznań University of Life Sciences (Poznań, Poland) with respect to animal experimentation and care of the animals under study, and all efforts were made to minimize suffering.

Birds and Housing

In two independent experiments, broiler chickens were given feed supplemented with different ionophore coccidiostats (salinomycin and monensin supplementation). In both experiments, a total of 400 one-day-old female Ross 308 chicks were randomly distributed to four dietary treatments, with 10 replicate pens per treatment and 10 birds per pen.

The first experiment was carried out to investigate the growth performance, selected blood parameters, and endogenous enzyme concentrations in birds fed diets supplemented with nisin or salinomycin. In the second experiment, the effects of monensin and nisin on growth performance, apparent ileal digestibility of crude protein and ether extracts, tibiotarsus mineralization, selected immunological organ weights, and selected gastrointestinal tract organ weights and lengths were examined. The housing conditions were the same in both experiments. The birds were kept in floor pens (1.00 × 1.00 m) over 35 d. Stock density was established at 10 birds/m^2. The birds were given 23 h of light and 1 h of dark during the first week and then 19 h of light and 5 h of dark from 7 to 21 d of age. From 22 to 35 d of age, there was 23 h of light and 1 h of dark.

Diets and Feeding Program

The composition of the experimental basal diets is shown in Table 1. In both experiments, the birds were fed ad libitum for 35 d. The experimental diets were designed to provoke gastrointestinal tract colonization

Table 1. Composition of the experimental diets.

Ingredient (g kg^{-1})	1 to 14 d	15 to 35 d
Wheat	456	475
Rye	100	100
Rapeseed meal	100	100
Soybean meal	234	199
Fish meal	20.0	20.0
Pig lard	57.0	81.1
Mineral-vitamin premix[a]	3.0	3.0
Dicalcium phosphate	19.4	12.4
Limestone	1.0	1.6
Salt (NaCl)	1.5	1.6
Sodium carbonate (Na_2CO_3)	1.5	1.0
L-Lysine	2.2	1.8
DL-Methionine	3.2	2.7
L-Threonine	1.4	1.5
Titanium oxide (TiO_2)[b]		2.0
Calculated nutritive value (g kg^{-1})		
ME (MJ kg^{-1})	12.6	13.3
Crude protein	216	201
Crude fat	72.3	96
Crude fiber	33.5	32.5
Sodium—Na (%)	1.6	1.4
Calcium—Ca (%)	8.5	7.0
Total P (%)	8.4	7.0
Lysine (%)	12.5	11.3
Methionine (%)	6.2	5.5
Methionine + cysteine (%)	9.9	9.1
Threonine (%)	9.2	8.7

[a]Provided the following per kilogram of diet: vitamin A, 11,166 IU; cholecalciferol, 2,500 IU; vitamin E, 80 mg; menadione, 2.50 mg; vitamin B_{12}, 0.02 mg; folic acid, 1.17 mg; choline, 379 mg; D-pantothenic acid, 12.50 mg; riboflavin, 7.0 mg; niacin, 41.67 mg; thiamine, 2.17 mg; D-bioyin, 0.18 mg; pyridoxine, 4.0 mg; ethoxyquin, 0.09 mg; Mn (MnO_2), 73 mg; Zn (ZnO), 55 mg; Fe ($FeSO_4$), 45 mg; Cu ($CuSO_4$), 20 mg; I (CaI_2O_6), 0.62 mg; and Se (Na_2SeO_3), 0.3 mg.

[b]Replaced corresponding amount of the wheat in each diet, from 30 to 35 d of broiler growth.

by *Clostridium perfringens* via the use of viscous cereals (wheat/rye), animal fat (pig lard), and fish meal in the diet. The diets were prepared in mash form; all of the raw materials were ground by disc mill (Skiold A/S, Denmark) at a 2.5-mm disc distance and mixed with any heat treatment. The diets were produced in the Piast Pasze feed mill (Lewkowiec, Poland) according to ISO 9001:2008 procedures. The feed was prepared on a laboratory-scale line equipped with a horizontal double band mixer (Zuptor, Gostyń, Poland) with roller mills (Skiold, Sæby, Denmark). Starter diets were offered to all birds from 1 to 14 d of age, and grower–finisher diets were offered from 15 to 35 d of age. Until 14 d of age, all birds were fed the same basal diet without titanium dioxide (TiO_2). In the last 5 d (30 to 35 d) of the experiment, 0.2% of the wheat was replaced by titanium dioxide as an internal marker for calculation of nutrient digestibility. No exogenous enzymes were used in the studies. The following treatments were applied: experiment 1: NA—no additives, SAL—salinomycin addition (60 mg/kg diet), NIS—nisin preparation (2,700 IU/kg diet), SAL+NIS—salinomycin (60 mg/kg diet) and nisin (2,700 IU/kg diet); experiment 2: NA—no additives, MON—monensin addition (100 mg/kg diet), NIS—nisin (2,700 IU/kg diet) and MON+NIS—monensin (100 mg/kg diet) and nisin (2,700 IU/kg diet).

Preparation of Nisin and Analysis of Nisin Concentrations

Nisin was prepared according to the method developed at the Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, using *Lactococcus lactis* subsp. *lactis* (ATCC 11,454). All details regarding preparation and concentration analyses of nisin were reported previously by Józefiak et al. (2013). The nisin activity expressed in international units (IU) was measured by a spectrophotometer (model Specord 205, Analytik Jena, Jena, Germany), and the results were compared to a commercially available nisin standard (Sigma, 1,000 IU/mg of solid) and converted into IU by equivalent (1 μg of nisin corresponds to 40 IU).

Data and Sample Collection

In both experiments, the following variables were analyzed on days 14 and 35: BWG, FI, and feed conversion ratio (FCR). At the end of the experiments (35 d), all of the chickens were killed by cervical dislocation. During dissection, the digesta from the duodenum and ileum was gently squeezed by segment from 10 individual birds (chosen at random from 10 replications per group), for analyses of pancreatic enzyme activity (experiment 1) and apparent ileal digestibility (experiment 2). Furthermore, in the first experiment, blood samples were collected from one bird per replicate pen (chosen at random) by puncture of the wing vein. Serum was obtained by centrifugation (Micro 220R, Hettich, Tuttlingen, Germany) at 1,000 × *g* at 8°C for 10 min and stored at −20°C until analysis. The following blood levels were determined: non-esterified fatty acid (**NEFA**), glucose, triglycerides (**TG**), cholesterol, total protein and albumin, alanine aminotransferase (**ALT**), and aspartate aminotransferase (**AST**). The serum concentration of insulin, glucagon, and leptin and the activity of lipase, amylase, and trypsin in the duodenal digesta were analyzed. The methods used for serum analyses are described in the next section.

In the second experiment, the apparent ileal digestibility of crude protein and ether extracts were measured. Furthermore, the following parameters were examined: crude ash, calcium, and phosphorus content of the tibiotarsus and selected immune organs weights in relation to body weight (BW) (% of BW), i.e., the thymus, spleen and bursa of Fabricius. The relative weights (% of BW) of selected gastrointestinal tract organs, i.e., duodenum, jejunum, ileum, caeca and pancreas, and their lengths in relation to BW (cm/kg BW), except for that of the pancreas, were measured. The jejunum was considered to begin at the duodenum and end at Meckel's diverticulum. The ileum was defined as the small intestinal segment caudal to Meckel's diverticulum. After dissection, the above–mentioned organs were rinsed in sterile water, drained and weighed using

PS 600/C/2 – Radwag (Radom, Poland) precision scales and then measured. For analysis of tibiotarsus mineralization, the right tibias from 10 birds per treatment (1 bird/pen, randomly chosen) were removed and frozen (−20°C) for further analyses according to the method described by Ptak et al. (2013).

Analyses of Blood Serum Parameters and Pancreatic Enzymes

The concentration of NEFA was measured according to the colorimetric method described by Duncombe (1964). Glucose concentration was analyzed colorimetrically using a Synergy 2 Multi-Mode Reader (BioTek Instruments, Inc., Winooski, VT, USA) with a Pointe Scientific kit reagent. Cholesterol and TG were measured using commercial (Pointe Scientific, Warsaw, Poland) enzymatic kits. Total protein and albumin were determined according to the methods described by Szymeczko et al. (2008). The activities of ALT and AST were analyzed using commercial (Pointe Scientific, Canton, OH) kits. Insulin, glucagon, and leptin concentrations were determined using commercial (Merck Millipore, Darmstad, Germany) radioimmunoassay kits according to the manufacturer's instructions.

The activity of lipase, amylase, and trypsin in the duodenal digesta was measured using BioVision colorimetric assay kits (Milpitas, CA). The digesta were homogenized on ice, and Tris buffered saline (TBS) was added to obtain 20% homogenates. Subsequently, the homogenates were centrifuged at 10,000 × *g* for 30 min at 4°C, and the supernatants were diluted 100 times using TBS. The incubation conditions were as described in detail by Pruszyńska–Oszmałek et al. (2015).

The tibiotarsus bones were cleaned of adherent tissue and ashed at 550°C for 14 h (Muffle Furnance P–300 5, Noberthem GmbH, Lilienthal, Germany). The ash weight was calculated relative to the tibial dry weight. The concentrations of calcium and phosphorus in the tibiotarsus were determined according to Ptak et al. (2013) using atomic absorption spectrophotometry (Varian Techtron AA 475, Pty. Ltd. Springvale, Australia).

Calculations and Statistical Analyses

The apparent ileal digestibility of crude protein and crude fat was calculated relative to the ratio of titanium dioxide (dietary marker) to the nutrient content in feed or excreta. The following equation was used (fat digestibility calculation as an example):

$$\begin{aligned}&\text{Fat digestibility (\%)}\\&= \left(1-\left[\left(\text{TiO}_2\%_{\text{diet}}/\text{TiO}_2\%_{\text{digesta/excreta}}\right)\right.\right.\\&\quad\left.\left.\times\left(\text{Fat}\%_{\text{digesta/excreta}}/\text{Fat}\%_{\text{diet}}\right)\right]\right)\times 100\end{aligned}$$

The immune organ index was calculated as the immune organ weight (g) divided by the chicken's weight before slaughter (Zhu et al., 2015).

The design of the experiments was completely randomized, and data were tested using the GLM procedure in SAS software. In both experiments, a two-factorial design was applied according to the following general model:

$$Y_{ij} = \mu + \alpha_i + \beta_j + (\alpha\beta)_{ij} + \delta_{ij}$$

where Y_{ij} is the observed dependent variable; μ is the overall mean; α_i is the effect of salinomycin or monensin; β_j is the effect of nisin; $(\alpha\beta)_{ij}$ is the interaction between salinomycin or monensin and nisin; and δ_{ij} is random error.

In cases in which the overall effect was significant ($P < 0.05$), the means were compared pairwise (pdiff). The results are given as the least squares means with pooled standard deviation.

RESULTS

Bird Performance

No mortality was observed in either experiment. In the first experiment, interactions between nisin and salinomycin were observed within days 1 to 14 for the case of BWG ($P < 0.01$) and FCR ($P = 0.01$) (Table 2). Nisin supplementation increased BWG in comparison to the NA and SAL diets ($P < 0.01$). However, there were no significance differences between NIS and SAL+NIS treatments in terms of BWG. The lowest BWG was observed in the NA group. NIS and SAL+NIS improved FCR on days 1 to 14 ($P < 0.01$). Nisin supplementation decreased the FCR by more than 10% in the first experimental period compared to the NA group. There were no statistically significant differences between treatments in terms of FI in the first period. In days 15 to 35, there was an interaction between salinomycin and nisin in BWG only ($P = 0.03$). The main effects of salinomycin and nisin were observed in terms of increasing BWG. The diet without additives resulted in the lowest BWG ($P < 0.01$). There were no statistical differences among the other groups. Salinomycin ($P = 0.02$) and nisin ($P < 0.01$) resulted in an increase in FI, but only salinomycin positively affected FCR ($P = 0.04$) on days 15 to 35. In the entire experiment, the interaction between these additives was significant for BWG alone ($P = 0.03$). Both salinomycin ($P < 0.01$) and nisin ($P < 0.01$) increased BWG; however, no synergistic or additional effects were observed. A mixture of salinomycin and nisin resulted in an increase of approximately 8% in BWG in comparison to the NA treatment ($P < 0.01$). Moreover, the effect of nisin supplementation on the increase in FI ($P < 0.01$) and the improvement of FCR by salinomycin

Table 2. The effect of dietary supplementation with nisin alone or in combination with salinomycin on the growth performance of broiler chickens (experiment 1).[1]

Treatment		Performance								
		1 to 14 d			15 to 35 d			1 to 35 d		
Salinomycin	Nisin	BW gain, g	FI, g	FCR, g:g	BW gain, g	FI, g	FCR, g:g	BW gain, g	FI, g	FCR, g:g
-	-	369[c]	574	1.56[a]	1,590[b]	2,635[b]	1.66	2,034[b]	3,211[b]	1.58
+	-	389[b]	572	1.47[b]	1,713[a]	2,732[a]	1.60	2,150[a]	3,297[a]	1.54
-	+	423[a]	584	1.39[c]	1,709[a]	2,757[a]	1.61	2,159[a]	3,342[a]	1.55
+	+	413[a]	579	1.40[c]	1,749[a]	2,795[a]	1.60	2,194[a]	3,374[a]	1.54
Pooled SEM		4.10	3.00	0.02	13.2	16.5	0.01	13.2	17.7	0.02
Model *P*		<0.01	0.5	<0.01	<0.01	<0.01	0.07	<0.01	<0.01	0.07
Model RMSE[2]		15.2	18.8	0.06	59.4	88.9	0.06	57.9	96.1	0.04
Interaction terms salinomycin × nisin		<0.01	0.8	0.01	0.03	0.3	0.2	0.03	0.4	0.2
Main effects										
Salinomycin										
None		397	580	1.47	1,650[b]	2,696[b]	1.64[a]	2,096[b]	3,276	1.56[a]
60 ppm		401	576	1.44	1,731[a]	2,763[a]	1.60[b]	2,172[a]	3,336	1.54[b]
Nisin										
None		379[b]	573	1.51[a]	1,654[b]	2,684[b]	1.63	2,092[b]	3,254[b]	1.56
2,000 IU/kg		418[a]	582	1.39[b]	1,729[a]	2,776[a]	1.61	2,176[a]	3,358[a]	1.54
P-value										
Salinomycin		0.3	0.5	0.1	<0.01	0.02	0.04	<0.01	0.06	0.04
Nisin		<0.01	0.2	<0.01	<0.01	<0.01	0.3	<0.01	<0.01	0.3

[a–c]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 10 chicks each.
[2]RMSE, root mean square error.

supplementation ($P = 0.04$) were observed in the entire experiment.

In the second trial, no interaction between monensin and nisin was recorded on days 1 to 14 (Table 3). The main effect of monensin addition did not affect growth performance in the first period. However, nisin supplementation improved BWG ($P < 0.01$) and FCR ($P < 0.01$). The differences in BWG and FCR between the NA and NIS treatments were 16% and 15%, respectively. On days 15 to 35, monensin ($P = 0.02$) and nisin ($P < 0.01$) significantly increased BWG and decreased FCR ($P < 0.01$). Supplementation with nisin increased FI ($P = 0.01$), while monensin supplementation had no effect ($P = 0.7$). Interaction between the experimental factors had a significant effect on FCR ($P < 0.01$). The highest FCR was recorded in the NA group ($P < 0.01$). Supplementation with MON, NIS, and MON+NIS decreased FCR to the same degree. In the entire experiment, monensin and nisin had significant effects on BWG ($P < 0.01$; $P < 0.01$) and FCR ($P < 0.01$; $P < 0.01$), while FI increased only with the addition of nisin ($P < 0.01$). Interaction between the experimental factors was observed for FCR ($P < 0.01$). The lowest values of FCR were observed in the NIS and MON+NIS treatments ($P < 0.01$). The combination of experimental factors reduced FCR by up to 6.5% in comparison to the NA group throughout the experiment.

Concentrations of Selected Blood Parameters

The concentrations of selected blood parameters are summarized in Tables 4 and 5. Hormone concentrations showed no statistically significant differences between treatments (insulin, $P = 0.3$; glucagon, $P = 0.8$; and leptin, $P = 0.6$). Furthermore, neither nisin nor salinomycin had an effect on any of the selected blood parameters, other than glucose (Table 5), the concentration of which decreased with the nisin diet ($P < 0.01$).

Activities of Endogenous Enzymes

The results for the activities of endogenous enzymes are shown in Table 6. An interaction between salinomycin and nisin was observed only for lipase ($P < 0.01$). Decreased lipase activity was recorded in the SAL and NIS treatments compared to the NA group.

Apparent Ileal Digestibility

No interactions were observed between monensin and nisin in terms of apparent ileal digestibility of crude protein and crude fat (Table 7). Nisin supplementation increased crude protein digestibility ($P = 0.01$). Supplementation with monensin and nisin (MON+NIS) and with NIS alone improved protein digestibility in comparison to the NA group ($P = 0.03$). There were no significant differences between the groups in terms of crude fat digestibility.

Tibiotarsus Mineralization

No significant interactions between monensin and nisin supplementation were observed with respect to crude ash, Ca, and P concentrations in the tibiotarsus

Table 3. The effect of dietary supplementation with nisin alone or in combination with monensin on the growth performance of broiler chickens (experiment 2).[1]

Treatment		Performance								
		1 to 14 d			15 to 35 d			1 to 35 d		
Monensin	Nisin	BW gain, g	FI, g	FCR, g:g	BW gain, g	FI, g	FCR, g:g	BW gain, g	FI, g	FCR, g:g
-	-	321[b]	581	1.82[a]	1,510[c]	2,548	1.69[a]	1,871[c]	3,129[b]	1.67[a]
+	-	327[b]	571	1.75[a]	1,580[b]	2,534	1.60[b]	1,946[b]	3,104[b]	1.60[b]
-	+	374[a]	579	1.55[b]	1,614[a,b]	2,593	1.61[b]	2,027[a]	3,172[a,b]	1.57[b,c]
+	+	376[a]	575	1.54[b]	1,641[a]	2,629	1.60[b]	2,076[a]	3,227[a]	1.56[c]
Pooled SEM		4.91	3.90	0.03	12.2	14.1	0.01	16.0	13.5	0.02
Model *P*		<0.01	0.8	<0.01	<0.01	<0.06	<0.01	<0.01	<0.01	<0.01
Model RMSE[2]		17.7	25.1	0.13	60.7	82.4	0.03	64.3	72.5	0.04
Interaction terms monensin × nisin		0.7	0.7	0.5	0.3	0.4	<0.01	0.5	0.1	<0.01
Main effects										
Monensin										
None		349	580	1.68	1,565[b]	2,572	1.65[a]	1,953[b]	3,152	1.62[a]
100 ppm		351	573	1.64	1,611[a]	2,582	1.60[b]	2,011[a]	3,166	1.58[b]
Nisin										
None		324[b]	575	1.78[a]	1,547[b]	2,541[b]	1.64[a]	1,911[b]	3,116[b]	1.63[a]
2,000 IU/kg		375[a]	577	1.54[b]	1,628[a]	2,611[a]	1.61[b]	2,052[a]	3,200[a]	1.56[b]
P-value										
Monensin		0.5	0.4	0.3	0.02	0.7	<0.01	<0.01	0.5	<0.01
Nisin		<0.01	0.9	<0.01	<0.01	0.01	<0.01	<0.01	<0.01	<0.01

[a–c]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 10 chicks each.
[2]RMSE, root mean square error.

Table 4. Serum concentrations of insulin, glucagon and leptin in broiler chickens fed diets supplemented with nisin alone or in combination with salinomycin over 35 d (experiment 1).[1]

Treatment		Hormones		
Salinomycin	Nisin	Insulin (ng/mL of digesta)	Glucagon (pg/mL of digesta)	Leptin (ng/mL of digesta)
-	-	0.95	339	1.70
+	-	1.19	360	2.24
-	+	1.18	356	2.63
+	+	1.15	369	2.34
Pooled SEM		0.07	10.1	4.30
Model *P*		0.6	0.7	0.8
Model RMSE[2]		0.54	62.8	2.07
Interaction terms salinomycin × nisin		0.3	0.8	0.6
Main effects				
Salinomycin				
None		1.07	348	2.23
60 ppm		1.17	364	2.30
Nisin				
None		1.07	351	1.92
2,000 IU/kg		1.16	362	2.49
P-value				
Salinomycin		0.5	0.3	1.0
Nisin		0.5	0.5	0.4

[1]Means represent 10 pens of 1 bird each.
[2]RMSE, root mean square error.

(Table 8). However, both supplements decreased calcium levels in the tibia ($P < 0.01$; $P = 0.04$).

Weights and Lengths of Selected Organs

The weights of selected immune organs are shown in Table 9. There were no interactions between monensin and nisin supplementation regarding the ratios of the weights of the thymus, spleen, or bursa of Fabricius to BW.

No significant interaction between monensin and nisin supplementation was observed with respect to the weights and lengths of selected gastrointestinal tract organs in relation to BW (Table 10). Monensin significantly reduced pancreas mass ($P < 0.01$), whereas nisin supplementation had no effect on this variable. Monensin supplementation also resulted in decreased jejunum weight ($P = 0.03$). Supplementation with nisin significantly reduced the duodenal, jejunal, and ileal weight and length and the caecal length.

Table 5. Concentrations of selected blood parameters in broiler chickens fed diets supplemented with nisin alone or in combination with salinomycin over 35 d (experiment 1).[1]

Treatment		Blood parameters							
Salinomycin	Nisin	NEFA[2] (mmol/L)	Glucose (mg/dL)	TG[3] (mg/dL)	Albumin (g/dL)	Total protein (g/dL)	Cholesterol (mg/dL)	ALT[4] (IU/L)	AST[5] (IU/L)
-	-	0.62	134[a]	87.6	2.20	4.09	160	3.05	35.6
+	-	0.61	132[a,b]	92.6	2.14	4.10	159	2.93	38.5
-	+	0.63	129[b]	88.7	2.14	4.02	157	3.58	41.2
+	+	0.62	129[b]	90.4	2.11	3.95	149	3.18	40.2
Pooled SEM		0.01	0.55	2.45	0.03	0.03	2.05	0.20	1.39
Model *P*		0.9	<0.01	0.9	0.6	0.3	0.2	0.7	0.5
Model RMSE[6]		0.07	3.78	19.2	0.20	0.25	15.6	1.52	10.8
Interaction terms salinomycin × nisin		0.7	0.2	0.8	0.7	0.5	0.4	0.7	0.5
Main effects									
Salinomycin									
None		0.63	131	88	2.17	4.05	158	3.32	38.5
60 ppm		0.62	130	92	2.13	4.03	154	3.05	39.4
Nisin									
None		0.62	133[a]	90	2.17	4.10	159	2.99	37.1
2,000 IU/kg		0.63	129[b]	90	2.12	3.98	153	3.38	40.7
P-value									
Salinomycin		0.6	0.4	0.5	0.4	0.7	0.3	0.5	0.7
Nisin		0.6	<0.01	0.9	0.4	0.08	0.1	0.3	0.2

[a,b]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 1 bird each.
[2]NEFA, Non-esterified fatty acid
[3]TG, Triglycerides.
[4]ALT, Alanine aminotransferase.
[5]AST, Aspartate aminotransferase.
[6]RMSE, root mean square error.

Table 6. Activities of selected endogenous enzymes in duodenal digesta of broiler chickens fed diets supplemented with nisin alone or in combination with salinomycin over 35 d (experiment 1).[1]

Treatment		Enzymes		
Salinomycin	Nisin	Lipase (U/L)	Amylase (U/L)	Trypsin (U/L)
-	-	343[a]	2,697	427
+	-	275[b]	2,605	415
-	+	276[b]	2,264	315
+	+	312[a,b]	2,536	423
Pooled SEM		8	326	21
Model *P*		<0.01	1.0	0.1
Model RMSE[2]		57	2,565	151
Interaction terms salinomycin ×nisin		<0.01	0.8	0.1
Main effects				
Salinomycin				
None		308	2,473	369
60 ppm		294	2,572	419
Nisin				
None		308	2,650	421
2,000 IU/kg		294	2,401	369
P-value				
Salinomycin		0.3	0.9	0.2
Nisin		0.4	0.7	0.2

[a,b]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 1 bird each.
[2]RMSE, root mean square error.

DISCUSSION

These studies confirmed the positive effects of nisin supplementation on broiler chickens performance (Józefiak et al., 2013; Kierończyk et al., 2016). Supplementation with nisin improved BWG and FCR values, especially in the first period (days 1 to 14). By day 35, nisin supplementation may increase BWG by 6% to more than 10%, depending on the experiment (Józefiak et al., 2013; Kierończyk et al., 2016). Nisin's positive influence on growth performance is closely related to nisin activity (Józefiak et al., 2013). Based on earlier studies, nisin is most effective at 2,500 to 2,700 IU/kg of feed. Supplementation with nisin may

Table 7. Apparent ileal digestibility of crude protein and ether extract in broiler chickens fed diets supplemented with nisin alone or in combination with monensin over 35 d (experiment 2).[1]

Treatment		Apparent ileal digestibility	
Monensin	Nisin	Crude protein	Ether extract
-	-	55.5[a,b]	72.5
+	-	49.8[b]	72.1
-	+	59.0[a]	76.0
+	+	57.4[a]	78.1
Pooled SEM		1.28	3.94
Model *P*		*0.03*	*0.5*
Model RMSE[2]		4.55	6.43
Interaction terms		0.4	0.7
monensin × nisin			
Main effects			
Monensin			
None		57.5	74.5
100 ppm		53.6	75.1
Nisin			
None		52.3[b]	72.3
2,000 IU/kg		58.2[a]	77.0
P-value			
Monensin		0.1	0.8
Nisin		0.01	0.1

[a,b]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 birds in 5 pooled replicates.
[2]RMSE, root mean square error.

Table 8. Crude ash, calcium, and phosphorus concentration in the tibiotarsus (experiment 2).[1]

Treatment		Tibiotarsus		
Monensin	Nisin	Crude ash	Ca	*P*
-	-	31.1	36.6[a]	11.0
+	-	31.6	35.3[b]	11.1
-	+	29.9	36.4[a,b]	11.1
+	+	29.7	33.7[c]	11.0
Pooled SEM		0.67	0.25	0.17
Model *P*		0.4	<0.01	0.5
Model RMSE[2]		3.44	1.55	0.21
Interaction terms		0.6	0.07	0.2
monensin × nisin				
Main effects				
Monensin				
None		30.5	36.5[a]	11.1
100 ppm		30.7	34.5[b]	11.0
Nisin				
None		31.4	35.9[a]	11.1
2,000 IU/kg		29.8	35.0[b]	11.0
P-value				
Monensin		0.9	<0.01	0.6
Nisin		0.09	0.04	0.6

[a–c]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 1 bird each.
[2]RMSE, root mean square error.

reduce FCR by 2 to 5% in comparison to the NA group. Changes in selected microbiota populations in response to nisin and salinomycin supplementation are consistent with the observed improvement in growth performance. Kierończyk et al. (2016) demonstrated that supplementation with nisin and salinomycin decreased the total number of bacteria, Enterobacteriacae, *Clostridium perfringens*, and *Lactobacillus* spp./*Enterococcus* spp. This positive effect involves a reduction in competition between host-bird and GIT microbiota for nutrients and an increase in fat digestibility by limiting of bile salt deconjugation (Masuda, 1981; Klaver and Meer, 1993). As this study shows, nisin and salinomycin have highly similar effects on growth performance, whereas nisin supplementation, compared to monensin supplementation, increased BWG over the entire experimental period. Moreover, these results suggest that supplementation with monensin supported by nisin may result in enhanced rearing efficiency. Similar results were found for ether extract digestibility, which was improved by the addition of both alimentary factors by up to 7.5%. In general, nutrient digestibility is reflected in the birds' growth performance. Nisin supplementation improved crude protein and ether extract

Table 9. Weights of selected immune organs in relation to body weight (experiment 2).[1]

Treatment		Immune organs index		
Monensin	Nisin	Thymus (% of BW)	Spleen (% of BW)	Bursa (% of BW)
-	-	0.27	0.12	0.13
+	-	0.27	0.11	0.14
-	+	0.26	0.12	0.16
+	+	0.26	0.12	0.12
Pooled SEM		0.01	0.01	0.02
Model *P*		0.9	0.9	0.4
Model RMSE[2]		0.05	0.03	0.04
Interaction terms				
monensin × nisin		0.9	0.7	0.4
Main effects				
Monensin				
None		0.26	0.12	0.14
100 ppm		0.26	0.12	0.14
Nisin				
None		0.27	0.12	0.13
2,000 IU/kg		0.26	0.12	0.15
P-value				
Monensin		0.8	0.5	0.8
Nisin		0.5	0.7	0.2

[1]Means represent 10 pens of 1 bird each.
[2]RMSE, root mean square error.

Table 10. Influence of diets containing dietary nisin alone or in combination with monensin on the length (cm/kg BW) and weight (% of BW) of selected sections of the gastrointestinal tract (experiment 2).[1]

Treatment		Length (cm/kg BW)				Weight (% of BW)				
Monensin	Nisin	duodenum	jejunum	ileum	caecum	duodenum	jejunum	ileum	caecum	pancreas
-	-	16.8	40.1^{a}	42.1^{a}	9.1^{a,b}	0.69^{a}	1.48^{a}	1.07^{a}	0.29	0.25^{a}
+	-	16.5	39.6^{a}	42.2^{a}	9.8^{a}	0.71^{a}	1.39^{a}	1.06^{a}	0.32	0.21^{b}
-	+	16.2	38.5^{a}	38.7^{a,b}	8.9^{a,b}	0.65^{a,b}	1.27^{b}	0.97^{a,b}	0.31	0.23^{a,b}
+	+	15.0	34.7^{b}	35.9^{b}	8.4^{b}	0.61^{b}	1.16^{b}	0.93^{b}	0.28	0.21^{b}
Pooled SEM		0.26	0.69	0.71	0.23	0.01	0.03	0.02	0.01	0.01
Model *P*		0.08	0.02	<0.01	0.04	<0.01	<0.01	0.01	0.2	<0.01
Model RMSE[2]		1.92	5.00	4.89	1.37	0.09	0.17	0.13	0.06	0.03
Interaction terms		0.4	0.2	0.3	0.1	0.1	0.9	0.6	0.06	0.4
monensin × nisin										
Main effects										
Monensin										
None		16.5	39.3	40.3	9.0	0.67	1.37^{a}	1.02	0.30	0.24^{a}
100 ppm		15.7	37.1	39.0	9.1	0.66	1.27^{b}	1.00	0.30	0.21^{b}
Nisin										
None		16.6^{a}	39.8^{a}	42.1^{a}	9.5^{a}	0.70^{a}	1.44^{a}	1.07^{a}	0.31	0.23
2,000 IU/kg		15.6^{b}	36.6^{b}	37.3^{b}	8.6^{b}	0.63^{b}	1.21^{b}	0.95^{b}	0.29	0.22
P-value										
Monensin		0.2	0.09	0.3	0.8	0.6	0.03	0.5	1.0	<0.01
Nisin		0.05	0.02	<0.01	0.02	<0.01	<0.01	<0.01	0.3	0.1

[a,b]Means within a column with no common superscripts differ significantly ($P < 0.05$).
[1]Means represent 10 pens of 1 bird each.
[2]RMSE, root mean square error.

digestibility by 11% and 6.5%, respectively. There are little data about nutrient retention or digestibility in terms of bacteriocin administration in broiler diets. The usage of divercin AS7, the bacteriocin produced by *Carnobacterium divergens* AS7, at various levels (200 AU/mL vs. 1,600 AU/mL) did not influence nitrogen retention or apparent total tract crude fat digestibility (Józefiak et al., 2010). The liquid and lyophilized forms of this bacteriocin do not affect the ileal digestibility of nutrients (Józefiak et al., 2011a,b; Józefiak et al., 2012).

There is, however, a lot of information about the effects of alimentary factors, including antibiotics, on GIT measurements (Miles et al., 2006; Awad et al., 2009; Madej et al., 2015; Wang et al., 2016). In general, a reduced intestinal size indicates intensified nutrient absorption and utilization (Dibner and Richards, 2005). Thus, the saved energy can be used for growth or enhanced immune status (Kim et al., 2011). In this study, nisin supplementation had a significant effect on reducing the weights and lengths of small intestine segments and improving growth performance and digestibility. These results are also in agreement with the results of De Verdal et al. (2010), who found that birds bred for high digestive efficiency were characterized by decreased weights and lengths of intestinal segments.

Improved digestive function could also be the result of the histological structure of the GIT epithelium. A large body of data suggest that probiotics induce an increase in surface area, which is correlated with better nutrient absorption (Tsirtsikos et al., 2012; Rawski et al., 2016). In bacteriocins, nisin administered to rabbits did not influence intestinal morphometry, i.e., the villus height/crypt depth ratio (Lauková et al., 2014). Józefiak et al. (2012) demonstrated that the positive effect of divercin AS7 supplementation on GIT histomorphology is dependent on health status. For these reasons, it is important to consider continuing experiments on the effect of nisin on GIT histomorphology in broiler chickens.

In general, little is known about the influence of bacteriocins or bacteriocinogenic probiotic strains on the secretion of pancreatic enzymes. Pruszyńska–Oszmałek et al. (2015) found that *Lactococcus lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* injected in ovo significantly improve the total activity of pancreatic enzymes, i.e., amylase, lipase, and trypsin. Palamidi et al. (2016) proved that a multispecies probiotic combination (*Lactobacillus reuteri*, *Enterococcus faecium*, *Bifidobacterium animalis*, *Pediococcus acidilactici*, and *Lactobacillus salivarius*) increased nutrient digestibility. On the other hand, the results presented in this study show that separate administration of nisin and salinomycin decreases lipase activity. There was no effect on the other endogenous enzymes examined. The addition of salinomycin was associated with inhibition of lipase activity (Engberg et al., 2000). However, to our knowledge, this experiment is the first to show an influence of nisin supplementation on the activities of digestive enzymes.

The concentrations of insulin, glucagon, and leptin were not affected by nisin supplementation. There are no data in the literature on the impact of bacteriocins on hormones controlling the function of the GIT or on avian metabolism. Leptin decreases feed intake in broilers via effects on the central nervous system (Denbow et al., 2000). As this study shows, nisin did not adversely affect the serum concentrations of leptin, insulin or glucagon, as supported by the growth performance data.

There is no information in the literature about the effect of nisin on broiler chicken blood parameters. However, there is some information from in vivo trials using various forms of administration in rabbits, rats and mice. Lauková et al. (2014) conducted an experiment on rabbits (nisin 500 IU–20 μg/animal/day; administered in the drinking water), which resulted in no significant differences in total protein, TG, cholesterol, glucose, calcium, or ALT. Moreover, intravaginal application (50 mg/day) does not alter blood parameters, i.e., glucose, total protein, albumin, ASP and ALT (Reddy et al., 2004). Aranha et al. (2004) confirmed that nisin affects neither the above-mentioned blood parameters nor total cholesterol. However, as demonstrated by Hagiwara et al. (2010), animal sex is especially important for the effects of nisin on blood parameters. In general, males do not demonstrate any significant differences in serum biochemistry, while in females, nisin (oral administration) may decrease total cholesterol and TG and increase albumin concentration. In this study, only the glucose concentration was lowered by dietary nisin in comparison to the NA treatment. Nisin supplementation does not appear to negatively affect blood parameters in broilers.

Tibiotarsus health is one of the most important issues in modern poultry production and management (Kierończyk et al., 2017). As this study shows, nisin did not affect crude ash and phosphorus contents but did affect calcium in the tibia. However, nisin administered simultaneously with monensin may change the mineralization of the bones in terms of the calcium content. The research of Orriss et al. (2013) has shown that toxic monensin activity in osteoblasts resulted in cell death. Moreover, monensin can disrupt collagen secretion (Rath, 2004). On the other hand, there is no experimental evidence that monensin affects tibial dyschondroplasia or tibia calcium in broiler chickens (Edwards, 1985; Ward et al., 1990). It is difficult to explain why monensin and nisin decreased calcium levels. The calcium level found in this study is in the normal range recorded by Ptak et al. (2013) and Nkukwana et al. (2014). The low phosphorus content may be explained by the lack of supplemental phytase in the diet (Jiang et al., 2013). No leg abnormalities were recorded during the experiments.

The immune status of the host bird plays a crucial role in infectious disease prevention. The thymus and the bursa of Fabricius are primary lymphoid tissues, and the spleen is a secondary lymphoid tissue (Kierończyk et al., 2016). Their weight in proportion to the chicken's weight or the absolute organ weight is commonly used to qualify the immune status of the birds (Tong et al., 2014; Zhu et al., 2015). In this study, there was no effect of nisin or monensin on the immunological organ index. Ogunbanwo et al. (2004) demonstrated that the addition of a bacteriocin produced by *Lacobacillus plantarum* F1 to the diet of broiler chickens challenged with *E. coli* 02: KH6 showed comparable weights of the spleens, livers, kidneys, and lungs with the uninfected control birds. Moreover, the use of probiotics has no significant impact on the relative weight of the spleen, though *Lactococcus garvieae* B301 application does have an effect on the thymus and bursa of Fabricius (Zhang et al., 2015). However, Poorghasemi et al. (2015) demonstrated that the bursa mass may change without an immune response. The relative weight of the bursa found in this study is similar to results found by Tong et al. (2015) and Kim et al. (2016). Bacteriocins including nisin appear to have no negative effect on the broiler chickens' immune status, but tests of immunoglobulin production or concentrations in serum are needed for greater certainty.

CONCLUSIONS

Supplementation of the broiler chickens' diet with nisin resulted in improved growth performance and apparent ileal digestibility of crude protein and reduced the length or weight of various GIT segments. No negative effect of nisin on tibiotarsus mineralization was found. However, supplementation did not result in changes in biochemical blood parameters. These results suggest that nisin may be used as a novel growth promoter with no negative effects on the birds' metabolism or immune status. Additionally, the combination of nisin with ionophores enhanced the effectiveness of monensin, though no synergistic effect of salinomycin and nisin could be confirmed over the 35-day course of this experiment.

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