



# The nisin improves broiler chicken growth performance and interacts with salinomycin in terms of gastrointestinal tract microbiota composition

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**ABSTRACT.** The aim of present study was to evaluate the potential synergistic effect of salinomycin and nisin on gastrointestinal tract microbial ecology and activity as well as the influence of nisin on broiler chicken growth performance. In the first experiment, which lasted 35 days, such dietary additions as: NA – no additives, SAL – salinomycin (60 mg · kg<sup>-1</sup> diet), NIS – nisin (2700 IU · kg<sup>-1</sup> diet) were used. Nisin addition in comparison to salinomycin and control treatments, improved body weight gain in the entire experiment (days 1–35) as well as increased feed intake and decreased feed conversion ratio, but only in the starter period (days 1–14). In the second experiment the same dietary additions and also SAL+NIS – salinomycin and nisin (60 mg · kg<sup>-1</sup> diet and 2700 IU · kg<sup>-1</sup> diet, respectively) were applied. The results of the second experiment indicated that salinomycin and nisin combination decreased the total bacteria counts, as well as *Enterobacteriaceae*, *Clostridium perfringens*, *Lactobacillus* spp./*Enterococcus* spp. and *Clostridium coccoides*–*Eubacterium rectale* cluster in the ileum. Furthermore, the interaction between applied factors was noticed in the decreasing total bacteria counts, *Lactobacillus* spp. *Enterococcus* spp., *Clostridium coccoides*–*Eubacterium rectale* cluster and increasing signals from *Bifidobacterium* spp. as well as *Streptococcus* sp. *Lactococcus*. There were no interactions between nisin and salinomycin in terms of organic acids concentration in the crop, gizzard, ileum and caecum, as well as pH value, except gizzard. The results of the present study have indicated the positive effect of nisin on broiler growth performance and the fact that nisin and salinomycin can act synergistically in scope of ileal microbiota ecology modification.

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## Introduction

In terms of health and growth performance, the modulation of gastrointestinal tract (GIT) microbiota is one of the most important issues of modern broiler

chicken nutrition. Wide spectrum of information is available about the influence of probiotics and ionophore coccidiostats on the endogenous chicken microbiota (Engberg et al., 2000; Alloui et al., 2013). However, less is known about bacteriocins,

which have been used for decades in human foods (Food and Drug Administration, 1988) and may exert important effects on GIT microecology.

Bacteriocins are relatively small peptides produced by bacterial cell ribosomes which have antimicrobial properties against closely related bacteria, other than the producing strain (Józefiak and Sip, 2013). However, only pediocin and nisin are used so far in the production of meat (Janes et al., 2002), vegetables (Vescovo et al., 1995) and milk products (Zottola et al., 1994), which limit food-borne diseases caused by e.g., *Listeria monocytogenes* or *Staphylococcus aureus*. Nisin consists of 34 amino acids and is the best known bacteriocin classified as lantibiotics (Class Ia) (Józefiak and Sip, 2013). Its antimicrobial function is directed against Gram-positive bacteria including *Clostridium perfringens* – one of the causal agents of necrotic enteritis (Riddell and Kong, 1992), and also Gram-negative bacteria (Boziaris and Adams, 1999). In 1983 the European Union designated it as E 234 and in 1988 it received the 'Generally Recognized As Safe' (GRAS) status in the USA. However, nisin has not been registered as a feed additive in Europe yet, and its usage in commercial animal nutrition is still not allowed.

Salinomycin, a ionophore coccidiostat, is commonly used against *Eimeria* spp. in broiler nutrition worldwide. The synergistic effects of this coccidiostat and other substances such as polymyxin B and doxorubicin were observed by Ogita et al. (2009) and Liffers et al. (2013). However, there are limited data concerning the impact of nisin and salinomycin combination on broiler GIT microbial ecology. Previous studies suggested, that salinomycin and nisin exert a similar mode of action in the GIT microbiome and on broiler growth performance (Józefiak et al., 2013). Therefore, the aim of the present study was to investigate the potential synergistic effect of salinomycin and nisin on microbial ecology in broiler chicken GIT as well as influence of nisin on broiler growth performance.

## Material and methods

### Preparation of nisin and analysis of nisin concentration

Nisin was prepared according to the method elaborated at the Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, using *Lactococcus lactis* subsp. *lactis* (ATCC 11454). All details regarding preparation and concentration analyses of nisin have been re-

ported previously by Józefiak et al. (2013). In the first experiment only, nisin was encapsulated in a fat matrix as described in detail by Józefiak et al. (2011). The second experiment was carried out without nisin encapsulation, as preliminary data showed that encapsulated nisin does not differ from nonencapsulated nisin in the case of broiler growth performance (unpublished data). The nisin activity expressed in international units (IU) was measured by a spectrophotometer (model Specord 205, Analytik Jena, Jena, Germany), the results were compared with a commercially available nisin standard (1000 IU · mg<sup>-1</sup> of solid, Sigma-Aldrich, St. Louis, MO, USA) and converted into IU by equivalent (1 µg of nisin corresponds to 40 IU).

### Birds and housing

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

Two experiments on broiler chickens fed diets differing in nisin and salinomycin supplementation were conducted. The first experiment was carried out to investigate the growth performance of birds fed diet supplemented with nisin or salinomycin. In the second experiment potential synergistic effect of salinomycin and nisin on microecology of the GIT was examined. The housing conditions were the same in both experiments. Birds were kept in floor pens (1.00 × 1.00 m and 1.00 × 2.5 m) over 35 and 21 days, in the experiment 1 and 2, respectively. Stock density was established on the 10 birds per m<sup>2</sup>. The lighting cycles (hours of light : hours of dark) were: 23 : 1 during the first week, 19 : 5 from day 7 to 21, and 23 : 1 from day 22 to 35. In the first experiment, a total of 420 one-day-old male Ross 308 chicks were randomly distributed to 3 dietary treatments, 14 replicate pens per treatment and 10 birds per pen. In the second experiment, 100 one-day-old female Ross 308 chicks were used. In each treatment 25 birds were defined as individual replicates and the birds were randomly allocated to 4 groups.

### Diets and feeding programme

In both experiments, the birds were fed basal diet (Table 1) with additives *ad libitum* for 35 or 21 days, in the experiment 1 and 2, respectively. The diets were prepared in a mash form. The experimental

**Table 1.** Composition and nutritive value of basal diet (Experiments 1 and 2)

Indices	
Ingredients, g · kg <sup>-1</sup>	
wheat	566.7
rye	100.0
soyabean meal	252.6
beef tallow	55.8
mineral-vitamin premix <sup>1</sup>	3.0
dicalcium phosphate	11.9
limestone	5.8
NaCl	2.9
Na <sub>2</sub> CO <sub>3</sub>	0.2
L-lysine HCl	3.4
methionine hydroxy analogue	2.8
L-threonine	0.6
Calculated nutritive value	
apparent metabolizable energy, MJ · kg <sup>-1</sup>	12.97
crude protein, g · kg <sup>-1</sup>	200.0

<sup>1</sup> provided per kg of diet: IU: vit. A (retinol) 11 166, cholecalciferol 2 500; mg: vit. E (alpha tocopherol) 80, menadione 2.50, cobalamin 0.02, folic acid 1.17, choline 379, D-pantothenic acid 12.50, riboflavin 7.0, niacin 41.67, thiamine 2.17, D-biotin 0.18, pyridoxine 4.0, ethoxyquin 0.09, Mn 73, Zn 55, Fe 45, Cu 20, I 0.62, Se 0.3

diets were designed to provoke GIT colonization by *Clostridium perfringens*, due to the use of rye and beef tallow. The treatments applied in the experiment 1: NA – no additives, SAL – salinomycin addition (60 mg · kg<sup>-1</sup> diet), NIS – nisin preparation (2700 IU · kg<sup>-1</sup> diet), and in the experiment 2: NA – no additives, SAL – salinomycin addition (60 mg · kg<sup>-1</sup> diet), NIS – nisin (2700 IU · kg<sup>-1</sup> diet) and SAL+NIS – salinomycin (60 mg · kg<sup>-1</sup> diet) and nisin (2700 IU · kg<sup>-1</sup> diet).

### Data and sample collection

In the first experiment body weight gain (BWG), feed intake (FI) and feed conversion ratio (FCR) were analysed on day 14 and 35. Moreover, in the second experiment, microbiota populations, pH and organic acids concentrations in digesta from crop, gizzard, ileum and caecum were determined. At the end of the second experiment (day 21), all chickens were killed by cervical dislocation. The digesta from the crop, gizzard, ileum and caecum were gently squeezed, pooled by segments for 5 birds (5 replications per group) and mixed, pH was measured and about 10 g was immediately packed, sealed in sterilized plastic bags and stored in -80 °C for further organic acids analyses and fluorescent *in situ* hybridization (FISH) analyses (ileal digesta only).

**Table 2.** Oligonucleotide probes

Target	Probe	Sequence (5' to 3')
<i>Bacteroides-Prevotella</i> cluster	Bacto303	CCAATGTGGGGGACCTT <sup>1</sup>
<i>Clostridium perfringens</i>	Cperf191	GTAGTAAGTTG-GTTTCCTCG <sup>1</sup>
<i>Enterobacteriaceae</i>	Enter1432	CTTTTGCAACCCACT <sup>1</sup>
<i>Lactobacillus</i> spp./ <i>Enterococcus</i> spp.	Lab158	GGTATTAGCAYCTGTTTCCA <sup>1</sup>
<i>Clostridium coccooides-Eu-bacterium rectale</i> cluster	Erec482	GCTTCTTAGTCARGTACCG <sup>2</sup>
<i>Clostridium leptum</i> subgroup	Clept1240	GTTTTTRTCAACGGCAGTC <sup>3</sup>
<i>Streptococcus/Lactococcus</i>	Strc493	GTTAGCCGTCCCTTTCTGG <sup>4</sup>
<i>Bifidobacterium</i> spp.	Bif228	GATAGGGACGCGACCCCAT <sup>5</sup>

<sup>1</sup>Józefiak et al. (2013); <sup>2</sup>Franks et al. (1998); <sup>3</sup>Sghir et al. (2000); <sup>4</sup>Franks et al. (1998); <sup>5</sup>Marteau et al. (2001)

### Analyses of pH and organic acids

The pH value was measured immediately after slaughter using a combined glass and reference electrode (VWR International, pH 1000L, Leuven, Belgium) in pooled digesta from 5 chickens in the crop, gizzard, ileum and caecum. The concentration of organic acids in the contents of the different gastrointestinal segments was determined by gas chromatography (Model 6890, Hewlett Packard, Agilent Technologies, Naerum, Denmark) according to Canibe et al. (2007).

### Microbial community analysis by fluorescent *in situ* hybridization (FISH)

All details of sample preparation and FISH analyses for bacteria enumeration from ileal digesta were described by Józefiak et al. (2013). The oligonucleotides probes used in this study are presented in Table 2.

### Statistical analysis

The experiments had a completely randomized design, and data was tested using the GLM procedure of SAS software (version 5.0, Iowa, USA). In the first experiment means were separated using a Duncans's one-way ANOVA equation:

$$Y_{ij} = \mu + \alpha_i + \delta_{ij}$$

where:  $Y_{ij}$  – observed dependent variable,  $\mu$  – overall mean,  $\alpha_i$  – effect of treatment,  $\delta_{ij}$  – random error.

In the second experiment two factorial design was applied according to the following general model:

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

where:  $Y_{ij}$  – observed dependent variable,  $\mu$  – overall mean,  $\alpha_i$  – effect of salinomycin,  $\beta_j$  – effect of nisin,  $(\alpha\beta)_{ij}$  – interaction between salinomycin and nisin,  $\delta_{ij}$  – random error.

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

## Results

No mortality was recorded in both experiments. In the first experiment, the supplementation of nisin increased body weight gain (BWG) in the starter period, as compared to SAL and NA diets ( $P < 0.0001$ ) (Table 3). The addition of salinomycin and nisin increased BWG ( $P = 0.047$ ) throughout days 14–35. In the entire experimental period BWG was significantly higher ( $P = 0.001$ ) in chickens fed diet supplemented with nisin. Significant differences in feed intake (FI) and feed conversion ratio (FCR) between groups were observed only in the first experimental period (days 1–14). Nisin supplementation increased FI ( $P = 0.001$ ) and improved FCR ( $P < 0.0001$ ) in comparison to both SAL and NA diets (Table 3).

**Table 3.** Performance of broiler chickens (Experiment 1)

Indices	Treatment <sup>1</sup>			Polled SEM <sup>2</sup>	P-value
	NA	SAL	NIS		
Body weight gain, g					
days 1–14	422 <sup>b</sup>	416 <sup>b</sup>	466 <sup>a</sup>	3.91	<0.0001
days 14–35	2075 <sup>b</sup>	2127 <sup>a</sup>	2136 <sup>a</sup>	11.07	0.047
days 1–35	2496 <sup>b</sup>	2543 <sup>b</sup>	2601 <sup>a</sup>	12.36	0.001
Feed intake, g					
days 1–14	540 <sup>b</sup>	537 <sup>b</sup>	562 <sup>a</sup>	3.17	0.001
days 14–35	3194	3193	3227	12.15	0.436
days 1–35	3734	3729	3789	13.52	0.137
Feed conversion ratio, g · g <sup>-1</sup>					
days 1–14	1.28 <sup>a</sup>	1.29 <sup>a</sup>	1.21 <sup>b</sup>	0.01	<0.0001
days 14–35	1.54	1.50	1.51	0.01	0.123
days 1–35	1.49	1.46	1.45	0.01	0.057

<sup>1</sup>NA – no additives; SAL – salinomycin (60 mg · kg<sup>-1</sup> diet); NIS – nisin (2700 IU · kg<sup>-1</sup> diet); <sup>2</sup>SEM – standard error of mean; <sup>ab</sup> – means with different superscripts within a row are significantly different

In the second experiment, the effect of salinomycin decreased ( $P < 0.0001$ ) total bacteria counts, *Enterobacteriaceae*, *Clostridium perfringens*, *Lactobacillus* spp./*Enterococcus* spp., *Clostridium coccooides*–*Eubacterium rectale* cluster, and increased *Clostridium leptum* subgroup and *Bifidobacterium* spp. (Table 4). Nisin increased only *Bacteroides*–*Prevotella* cluster ( $P = 0.01$ ) and *Bifidobacterium* spp. counts ( $P < 0.001$ ), whereas the numbers of *Clostridium leptum* subgroup, *Streptococcus/Lactococcus*, *Clostridium coccooides*–*Eubacterium rectale* cluster were not influenced significantly. Furthermore, the effect of nisin decreased ( $P < 0.0001$ ) total bacteria counts, *Enterobacteriaceae* ( $P = 0.05$ ), *Clostridium perfringens* ( $P = 0.040$ ) and *Lactobacillus* spp./*Enterococcus* spp. ( $P < 0.0001$ ). The positive interactions between applied factors (SAL+NIS) were noticed in decreasing total number of bacteria ( $P < 0.0001$ ), *Lactobacillus* spp./*Enterococcus* spp. ( $P = 0.015$ ), *Clostridium coccooides*–*Eubacterium rectale* cluster ( $P < 0.001$ ) and increasing signals from *Streptococcus/Lactococcus* ( $P < .001$ ) as well as *Bifidobacterium* spp. ( $P < .0001$ ). Simultaneously, the highest concentrations of *Streptococcus/Lactococcus* ( $P = 0.0028$ ) and *Bifidobacterium* spp. ( $P < 0.0001$ ) populations were observed in birds from separate SAL and NIS treatments, whereas in NA group the lowest values were detected. In the case of SAL and NIS addition in single way, the positive effect on *Bacteroides*–*Prevotella* cluster decrease ( $P = 0.0003$ ) was recorded. Contrary, the use of both additives affected microbiota negatively and enhanced the GIT colonization by *Bacteroides*–*Prevotella* cluster ( $P < 0.001$ ). There was no interaction between both experimental factors, in scope of population number of *Enterobacteriaceae*, as well as *Clostridium leptum* modification.

The effect of salinomycin on pH value was noticed in case of crop ( $P = 0.0001$ ) and ileum ( $P = <.001$ ) (Table 5). The salinomycin addition caused pH increase in above-mentioned segments. The nisin supplementation decreased pH value in the crop ( $P = 0.0001$ ), as well as caecum ( $P = 0.002$ ). Salinomycin and nisin were not shown to exert a synergistic effect in terms of the digesta pH values in crop, ileum and caecum. Only in gizzard the interaction between these additives was noticed ( $P = 0.015$ ). The lowest pH of gizzard digesta was observed in the SAL treatment ( $P = 0.01$ ). There were no significant differences in pH value among other NA, NIS and SAL+NIS treatments in this segment.

The highest bacterial fermentation activity was observed in crop and caecum (Tables 6 and 7). There were no significant interactions between nisin and salinomycin in case of organic acids concentration in none of GIT segments. The salinomycin addition to the diet decreased concentrations of acetic acid ( $P = 0.020$ ), lactic acid ( $P = 0.026$ ), succinic acid ( $P = 0.023$ ) and total organic acids ( $P = 0.017$ ) in the

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**Table 4.** Selected microbiota populations (log cfu · ml<sup>-1</sup> digesta) in the ileal digesta determined by DAPI staining and fluorescent *in situ* hybridization (FISH) (Experiment 2)

Indices	Treatments <sup>1</sup>				Pooled SEM <sup>2</sup>	Main effects				P-value		
	NA	SAL	NIS	SAL+NIS		SAL		NIS		treatments effect		interaction
						-	+	-	+	SAL	NIS	SAL x NIS
DAPI <sup>2</sup>	8.50 <sup>b</sup>	8.60 <sup>a</sup>	8.48 <sup>b</sup>	8.11 <sup>c</sup>	0.004	8.49 <sup>a</sup>	8.36 <sup>b</sup>	8.55 <sup>a</sup>	8.30 <sup>b</sup>	<.0001	<.0001	<.0001
<i>Enterobacteriaceae</i>	7.64	7.53	7.61	7.42	0.024	7.63 <sup>a</sup>	7.47 <sup>b</sup>	7.59 <sup>a</sup>	7.52 <sup>b</sup>	<.0001	0.05	0.28
<i>Bacteroides–Prevotella</i> cluster	7.63 <sup>b</sup>	7.54 <sup>c</sup>	7.60 <sup>bc</sup>	7.73 <sup>a</sup>	0.016	7.62	7.63	7.59 <sup>b</sup>	7.66 <sup>a</sup>	0.59	0.01	<.001
<i>Clostridium perfringens</i>	7.90	7.55	7.78	7.53	0.021	7.84 <sup>a</sup>	7.54 <sup>b</sup>	7.73 <sup>a</sup>	7.66 <sup>b</sup>	<.0001	0.04	0.16
<i>Lactobacillus</i> spp./ <i>Enterococcus</i> spp.	8.04 <sup>a</sup>	7.85 <sup>b</sup>	7.86 <sup>b</sup>	7.53 <sup>c</sup>	0.017	7.95 <sup>a</sup>	7.69 <sup>b</sup>	7.95 <sup>a</sup>	7.70 <sup>b</sup>	<.0001	<.0001	0.01
<i>Clostridium leptum</i>	7.58	7.60	7.49	7.61	0.023	7.53 <sup>b</sup>	7.61 <sup>a</sup>	7.59	7.55	0.04	0.29	0.17
<i>Streptococcus</i> / <i>Lactococcus</i>	7.46 <sup>c</sup>	7.66 <sup>a</sup>	7.61 <sup>ab</sup>	7.55 <sup>bc</sup>	0.028	7.54	7.60	7.56	7.58	0.10	0.52	<.001
<i>Clostridium coccoides–Eubacterium rectale</i> cluster	7.88 <sup>b</sup>	7.79 <sup>c</sup>	7.98 <sup>a</sup>	7.58 <sup>d</sup>	0.020	7.93 <sup>a</sup>	7.69 <sup>b</sup>	7.84	7.79	<.0001	0.11	<.0001
<i>Bifidobacterium</i> spp.	7.62 <sup>c</sup>	7.90 <sup>a</sup>	7.92 <sup>a</sup>	7.79 <sup>b</sup>	0.015	7.77 <sup>b</sup>	7.84 <sup>a</sup>	7.76 <sup>b</sup>	7.86 <sup>a</sup>	0.01	<.001	<.0001

<sup>1</sup>NA – no additives, SAL – salinomycin (60 mg · kg<sup>-1</sup> diet), NIS – nisin (2700 IU · kg<sup>-1</sup> diet), SAL+NIS – mixture of nisin and salinomycin (doses as in SAL and NIS); <sup>2</sup>DAPI – total number of bacteria determined by 4',6-diamidino-2-phenylindole staining; <sup>3</sup>SEM – standard error of the mean; <sup>ab</sup> – means with different superscripts within a row are significantly different for treatments and each main effect separately

**Table 5.** pH in crop, gizzard, ileum and caecal digesta (Experiment 2)

Indices	Treatments <sup>1</sup>				Pooled SEM <sup>2</sup>	Main effects				P-value		
	NA	SAL	NIS	SAL+NIS		SAL		NIS		treatments effect		interaction
						-	+	-	+	SAL	NIS	SAL x NIS
Crop	5.71	6.29	5.13	5.84	0.159	5.42 <sup>b</sup>	6.05 <sup>a</sup>	5.98 <sup>a</sup>	5.49 <sup>b</sup>	0.0001	0.0001	0.57
Gizzard	3.20 <sup>a</sup>	2.70 <sup>b</sup>	3.08 <sup>a</sup>	3.13 <sup>a</sup>	0.135	3.14	2.92	2.96	3.10	0.048	0.18	0.015
Ileum	6.48	6.87	6.58	6.82	0.086	6.53 <sup>b</sup>	6.84 <sup>a</sup>	6.67	6.70	0.0008	0.72	0.37
Caecum	6.51	6.63	6.13	6.30	0.135	6.32	6.46	6.57	6.22	0.18	0.002	0.802

<sup>1</sup>see Table 4; <sup>2</sup>SEM – standard error of the mean; <sup>ab</sup> – means with different superscripts within a row are significantly different for treatments and each main effect separately

**Table 6.** Organic acids concentrations in crop and gizzard digesta, µmol · g<sup>-1</sup> (Experiment 2)

Indices	Treatments <sup>1</sup>				Pooled SEM <sup>2</sup>	Main effects				P-value		
	NA	SAL	NIS	SAL+NIS		SAL		NIS		treatments effect		interaction
						-	+	-	+	SAL	NIS	SAL x NIS
Crop												
formic acid	ND <sup>3</sup>	0.71	2.06	0.74	0.34	1.03	0.73	0.30	1.40	0.547	0.103	0.131
acetic acid	6.96	4.04	16.81	8.24	1.59	11.86 <sup>a</sup>	6.44 <sup>b</sup>	5.68 <sup>b</sup>	12.52 <sup>a</sup>	0.020	0.009	0.220
propionic acid	0.29	0.19	0.44	0.33	0.31	0.37	0.27	0.25	0.38	0.552	0.464	0.981
DL-lactic acid	21.24	4.09	63.97	31.48	27.86	42.61 <sup>a</sup>	19.74 <sup>b</sup>	13.89 <sup>b</sup>	47.72 <sup>a</sup>	0.026	0.006	0.455
succinic acid	2.89	0.50	7.39	3.03	3.36	5.14 <sup>a</sup>	1.94 <sup>b</sup>	1.86 <sup>b</sup>	5.21 <sup>a</sup>	0.023	0.026	0.468
total organic acids	31.34	9.52	90.67	43.81	37.06	61.01 <sup>a</sup>	29.12 <sup>b</sup>	21.99 <sup>b</sup>	67.24 <sup>a</sup>	0.017	0.005	0.339
Gizzard												
acetic acid	1.98	1.93	2.83	2.33	0.63	2.58	1.96	2.44	2.16	0.069	0.365	0.386
DL-lactic acid	1.01	ND	3.72	1.02	1.89	2.36 <sup>a</sup>	0.58 <sup>b</sup>	0.58 <sup>b</sup>	2.37 <sup>a</sup>	0.024	0.032	0.274
total organic acids	7.62	1.94	6.05	2.99	3.56	6.84 <sup>a</sup>	2.54 <sup>b</sup>	5.18	4.52	0.020	0.686	0.423

<sup>1</sup>see Table 4; <sup>2</sup>SEM – standard error of the mean; <sup>3</sup>ND – not detected; <sup>ab</sup> – within main effects means in a row with different superscripts are significantly different for treatments and each main effect separately; In gizzard digesta in group NA it was also 3.46 µmol · g<sup>-1</sup> of formic acid, as well as 0.33 µmol · g<sup>-1</sup> of n-butyric acid was detected

**Table 7.** Organic acids concentrations in ileum and caecal digesta,  $\mu\text{mol} \cdot \text{g}^{-1}$  (Experiment 2)

Indices	Treatments <sup>1</sup>				Pooled SEM <sup>2</sup>	Main effects				P-value		
	NA	SAL	NIS	SAL+NIS		SAL		NIS		treatments effect		interaction SAL x NIS
						-	+	-	+	SAL	NIS	
<b>Ileum</b>												
acetic acid	3.86	4.06	3.62	3.71	1.26	3.74	3.86	3.95	3.67	0.848	0.708	0.939
DL-lactic acid	7.67	3.65	15.45	2.81	6.98	11.56 <sup>a</sup>	3.17 <sup>b</sup>	5.95	9.13	0.008	0.262	0.140
total organic acids	11.69	7.71	20.11	6.52	7.11	15.90 <sup>a</sup>	7.03 <sup>b</sup>	9.99	13.31	0.004	0.221	0.089
<b>Caecum</b>												
acetic acid	60.28	67.55	72.20	61.52	10.01	66.24	64.11	63.39	66.86	0.644	0.505	0.104
propionic acid	5.98	6.66	4.98	5.79	0.98	5.48	6.16	6.27	5.39	0.129	0.079	0.891
iso-butyric acid	0.38	0.48	0.40	0.31	0.17	0.39	0.38	0.42	0.36	0.980	0.543	0.349
n-butyric acid	8.82	9.19	12.26	9.52	4.18	10.54	9.36	8.96	10.89	0.577	0.418	0.516
n-valeric acid	0.88	1.08	0.87	0.79	0.16	0.88	0.92	0.97	0.83	0.480	0.074	0.063
succinic acid	19.76	16.61	20.29	17.57	7.30	20.03	17.16	18.41	18.93	0.410	0.882	0.952
total organic acids	97.99	102.82	113.03	95.50	14.5	105.51	98.64	100.06	104.27	0.351	0.580	0.160

<sup>1</sup>see Table 4; <sup>2</sup>SEM – standard error of the mean; <sup>3</sup>ND – not detected; <sup>ab</sup> – within main effects means in a row with different superscripts are significantly different for treatments and each main effect separately; In ileal digesta in group NIS it was also  $1.04 \mu\text{mol} \cdot \text{g}^{-1}$  of formic acid, in group NA  $0.17 \mu\text{mol} \cdot \text{g}^{-1}$  of propionic acid was detected. In the case of caecal digesta it was also  $1.29 \mu\text{mol} \cdot \text{g}^{-1}$  and  $0.57 \mu\text{mol} \cdot \text{g}^{-1}$  of formic acid was detected, in group NIS and SAL, respectively. Additionally, in the case of caecal digesta it was also  $1.9 \mu\text{mol} \cdot \text{g}^{-1}$  and  $1.45 \mu\text{mol} \cdot \text{g}^{-1}$  of DL-lactic acid was detected, in group NA and NIS, respectively.

crop, as well as lactic acid ( $P = 0.024$ ;  $P = 0.020$ ) and total organic acids ( $P = 0.008$ ;  $P = 0.004$ ) in the gizzard and ileum, respectively. Nisin increased fermentation of acetic acid ( $P = 0.009$ ), lactic acid ( $P = 0.006$ ), succinic acid ( $P = 0.026$ ) and the sum of organic acid ( $P = 0.005$ ) in the crop, as well as lactic acid ( $P = 0.032$ ) in the gizzard. In the case of caecal fermentation, both NIS as well as SAL treatments did not affect short-chain fatty acids composition and simultaneously no interaction between these factors was noticed. Calculations of the percentage concentration of selected organic acids i.e. acetic, butyric and propionic acid, were made (data not shown). There were no significant differences between treatments in all GIT segments. Acetic acid was predominant fermentation product in crop, gizzard and ileum. Its concentration was up to 100% of the sum of above-mentioned organic acids. In terms of caecal fermentation, the highest value of acetic acid (approx. 80%) was observed, then 12% of butyric acid and 8% of propionic acid.

## Discussion

The current study demonstrated a positive effect of salinomycin and nisin on broiler chicken growth performance and microbiota ecology. The results confirmed that microbiota modulation through ionophore coccidiostats is based on the suppression of Gram-positive pathogens such as *Clostridium perfringens* (Bjerrum et al., 2005), which causes necrotic enteritis in poultry, and by limiting prolif-

eration of *Bacteroides*, *Clostridiaceae*, *Enterobacteriaceae* and *Lactobacillus* spp./*Enterococcus* spp. which can compete with the host-bird for nutrients uptake as well as impair fat absorption through bile acid deconjugation (Masuda, 1981; Klaver and van der Meer, 1993; Czerwiński et al., 2012).

The knowledge about bacteriocin application in livestock nutrition, including poultry, has been limited and has been primarily focused on the preservation properties of these substances in the food industry (Teo and Tan, 2005). Moreover, in many experiments carried out on broiler chickens, where probiotic strains were applied, the potential production of bacteriocins by these microorganisms and their impact on the GIT homeostasis was not investigated as a key factor. Meanwhile, earlier studies conducted on different bacteriocins suggests that divercin AS7 may compensate the negative effects associated with the presence of *C. perfringens*, while nisin supplementation enhanced growth performance of the birds through modulation of GIT microecology (Józefiak et al., 2012, 2013).

Previous studies about bacteriocin application in broiler chicken diets showed nearly equal effects of nisin and salinomycin applied separately (Józefiak et al., 2013). In this work, the possible synergistic influence of these compounds was examined. Hitherto, combinations of different substances with bacteriocins were used for improving their preservation properties and food elongation storage period in food industry by microbiota limitation (Gálvez et al., 2007). The synergistic effects between nisin

and various substances were repeatedly observed. Combinations of ethanol (Brewer et al., 2002), monolaurin (Mansour et al., 1999), sucrose fatty acid esters – sucrose palmitate and sucrose stearate (Thomas et al., 1998) and reuterin (Arqués et al., 2004) with nisin reduced *L. monocytogenes*, *B. licheniformis* (vegetative cells and spores), *B. cereus* (cells and spores), *L. plantarum* and *Staphylococcus aureus* development, respectively. Supplementation of these complexes did not exert a negative effect on Gram-negative pathogens. Other studies suggest that combinations of two different bacteriocins could improve their antibacterial activity when compared to those used separately (Bouttefroy and Millière, 2000). However, there are no studies on potential synergistic effects of ionophores and bacteriocins. In the present study combination of salinomycin and nisin influenced GIT microbiota counts. Therefore, it is suggested that those two additives can have more significant antimicrobial activity when used as a mixture. It is well-known that the salinomycin activity is connected with disturbances in osmotic homeostasis in the bacteria cell body (Augustine et al., 1992). On the other hand, the target of nisin is the cytoplasmic membrane which is perforated and then all ions could permeate outside the microbe body (Józefiak and Sip, 2013). Moreover, it is possible that connection of these modes of actions and targets i.e. pathogens may explain the improved antibacterial function. Furthermore, *Lactobacilli* and *Bifidobacterium* spp. populations may affect fat absorption by bile salt deconjugation (high bile salt hydrolase activity) (Klaver and van der Meer, 1993). Consequently, their reduction positively improved fat digestibility and utilization in the broiler GIT. In this case, it is very useful to convert these results to practical use, because the synergistic mixture allows to reduce the required dosage of a single compound (Thomas and Isak, 2005).

In the present study the interaction between nisin and salinomycin was noticed only in pH value of gizzard. Furthermore, nisin supplementation decreased pH value in crop and caecum. Digesta pH was lowered in caecum after nisin supplementation, which confirmed the findings of Józefiak et al. (2013). Gálvez et al. (2007) observed, that the lower pH can enhance bacteriocins efficacy by the translocation of their molecules through the bacterial cell wall. Thus, decreasing of crop pH value may suggest that this GIT segment is the most important for microbiota suppression in chickens (Kierończyk et al., 2016). Moreover, the pH values were in agreement with organic acids concentrations which did

not differ due to nisin and salinomycin interactions. There were no synergistic effect between experimental factors as well. The microbial fermentation was enhanced in the crop and caecum. However, the highest organic acids concentrations in the crop, gizzard, ileum and caecum in birds fed diet with nisin addition were noticed. In this case, nisin may escalate D-lactic acid synthesis by growth promoting of lactic acid bacteria (LAB) microbiota in comparison to Józefiak et al. (2013). In the case of ileal digesta, the formic acid was detected in the nisin supplemented treatment, which may be the effect of high *Bifidobacteria*, as well as *Clostridia* concentration (Macfarlane and Gibson, 1995). Nevertheless, more detailed analyses should be performed to fully describe the microbiota activity.

## Conclusions

The present study suggests that in broiler chicken nutrition, both nisin and salinomycin can act synergistically by modulating microbiota in the GIT. It seems that bacteriocins addition to the broiler diets may be a novel strategy in poultry production, with the aim to control microbial pathogens and improve growth, feed efficiency and animal health.

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