Dietary Nisin Modulates the Gastrointestinal Microbial Ecology and Enhances Growth Performance of the Broiler Chickens

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Abstract

Due to antimicrobial properties, nisin is one of the most commonly used and investigated bacteriocins for food preservation. Surprisingly, nisin has had limited use in animal feed as well as there are only few reports on its influence on microbial ecology of the gastrointestinal tract (GIT). The present study therefore aimed at investigating effects of dietary nisin on broiler chicken GIT microbial ecology and performance in comparison to salinomycin, the widely used ionophore coccidiostat. In total, 720 one-day-old male Ross 308 chicks were randomly distributed to six experimental groups. The positive control (PC) diet was supplemented with salinomycin (60 mg/kg). The nisin (NI) diets were supplemented with increasing levels (100, 300, 900 and 2700 IU nisin/g, respectively) of the bacteriocin. The negative control (NC) diet contained no additives. At slaughter (35 days of age), activity of specific bacterial enzymes (α- and β-glucosidases, α-galactosidases and β-glucuronidase) in crop, ileum and caeca were significantly higher (P<0.05) in the NC group, and nisin supplementation decreased the enzyme activities to levels observed for the PC group. A similar inhibitory influence on bacterial activity was reflected in the levels of short-chain fatty acids (SCFA) and putrefactive SCFA (PSCFA) in digesta from crop and ileum; no effect was observed in caeca. Counts of Bacteroides and Enterobacteriaceae in ileum digesta were significantly (P<0.001) decreased by nisin and salinomycin, but no effects were observed on the counts of Clostridium perfringens, Lactobacillus/Enterococcus and total bacteria. Like salinomycin, nisin supplementation improved broiler growth performance in a dose-dependent manner; compared to the NC group, the body weight gain of the NI900 and NI2700 groups was improved by 4.7 and 8.7%, respectively. Our findings suggest that dietary nisin exerts a mode of action similar to salinomycin and could be considered as a dietary supplement for broiler chickens.

Introduction

Lactic acid bacteria (LAB) of the genera Lactococcus, Streptococcus, Pediococcus, Leuconostoc, Lactobacillus and Carnobacterium are the most commonly used starter cultures in food industry. Their mode of action is mainly based on the antimicrobial effect of lactic acid and pH reduction in combination, but certain LAB are capable of producing different types of bacteriocins, which are small peptides, lethal to bacteria other than the producing strain, but most often with a rather narrow target spectrum in comparison to antibiotics.

Bacteriocins are found in the gastrointestinal tract (GIT) of animals, in soil as well as in food, e.g. fermented milk products, cheese, or meat. The widespread use of bacteriocins in food industry is due to their antimicrobial activity against key Gram-positive pathogens of food-borne diseases, such as Listeria monocytogenes or Staphylococcus aureus. In contrast, there is so far only limited information on their usage and efficacy in feed industry [1].

In a series of our studies, we have recently demonstrated significant effects of bacteriocin - divercin AS7 - on the broiler chicken GIT microbiota and fermentation status as well as bird performance [2-5]. Moreover, other studies have shown dietary...
efficacy of other bacteriocins in broiler chickens [6,7]. However, to our knowledge there are no available data on in vivo usage and effects of nisin; probably the most explored and commonly used bacteriocin in many types of human foodstuffs.

Nisin is a 34 amino acid residues peptide, with a molecular mass of 3.5 kDa and classified as a class-Ia bacteriocin or lantibiotic [8]. Its usage in food is approved by the European Union with the assigned E-number E234 (EEC, 1983). In USA it has been granted GRAS (Generally Recognized As Safe; notice no. GRN 000065) status by the United States Food and Drug Administration. However, nisin is not included in the European Union Register of Feed additives (EU 1831/2003). Thus, even though this bacteriocin appears in many dairy products and active packing systems, its usage in animal nutrition is still forbidden.

A recent study by Udompijitkul et al. [9] indicated that nisin exerts strong antimicrobial activity in a meat model system against different isolates of *Clostridium perfringens*; an important poultry pathogen. Earlier studies conducted by Bernbom et al. [10] have shown that application of nisin-producing *Lactococcus lactis* strain to rat diets, affected the composition of the intestinal microbiota by increasing *Bifidobacterium* numbers and suppressing the enterococci streptococci population. The aim of the present study was therefore to evaluate the influence of dietary nisin on broiler chicken performance and GIT microbial ecology.

Material and Methods

Ethics statement

This study was carried out in strict accordance with the recommendations of the National Ethic Commission (Warsaw, Poland). All procedures and experiments complied with the guidelines and were approved by the Local Ethic Commission of the Poznań University of Life Sciences (Poznań, Poland) with respect to animal experimentation and care of animals under study, and all efforts were made to minimize suffering.

Birds and Housing

In total, 720 one-day-old male Ross 308 chicks were randomly distributed to 6 experimental groups using 12 replicate pens per treatment and 10 birds per pen. The broiler chickens were kept in floor pens (1.2 × 0.8 m) over a production period of 35 d. 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 d 7 to 21. From 22 to 42 d of age, there was 23 h of light and 1 h of dark.

Diets and Feeding Program

The composition of the experimental diet is shown in Table 1. The nisin activity was expressed in international activity unit (IU). The diet was formulated to stimulate the proliferation of *C. perfringens* by use of viscous cereals (barley/wheat), animal fats (beef tallow/pig lard), and fishmeal [11-13]. The diets were prepared in mash form; all raw materials were ground by disc mill (Skiodl A/S, Denmark) at 2.5 mm disc distance, mixed without any heat treatment, and fed *ad libitum* to the birds.

| Ingredients (g/kg) | Diet (1—35 d) |
|-------------------|---------------|
| Wheat             | 326.8 |
| Barley            | 250.0 |
| Soybean meal      | 215.4 |
| Beef tallow       | 30.0 |
| Pig lard          | 53.7 |
| Double zero rapeseed meal | 60.0 |
| Fish meal         | 30.0 |
| Monocalcium phosphate | 11.0 |
| Mineral-vitamin premix | 5.0 |
| Limestone         | 4.2 |
| L-Lysine -HCl     | 2.6 |
| DL-Methionine     | 2.1 |
| L-Threonine       | 0.3 |
| Sodium carbonate (Na₂CO₃) | 1.0 |
| Salt (NaCl)       | 2.6 |
| Titanium oxide (TiO₂) | 2.0 |
| ME (MkJ/kg)       | 12.95 |
| Crude protein     | 220.0 |
| Crude fat         | 100.0 |
| Crude fibre %     | 34.50 |
| Calcium - Ca %    | 8.50 |
| Lysine %          | 13.0 |
| Methionine %      | 5.5 |
| Methionine + Cystine % | 9.3 |
| Threonine %       | 8.1 |
| P available.      | 4.20 |
| Analysed composition (g/kg) | |
| Crude protein     | 214.0 |
| Crude fibre       | 39.2 |
| Crude fat         | 101.2 |
| ME (MkJ/kg)       | 12.89 |

Providing the following per kilogram of diet: vitamin A (retinol), 11,166 IU; cholecalciferol, 2,500 IU; vitamin E (alpha tocopherol), 80 mg; menadione, 2,500 mg; cobalamin, 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; thiamin, 2.17 mg; D-biotin, 0.18 mg; pyridoxine, 4.0 mg; ethoxyquin,0.09 mg; Mn (MnO₂), 73 mg; Zn (ZnO), 55 mg; Fe (FeSO₄)₂, 45 mg; Cu (CuSO₄), 20 mg; I (CaI₂), 0.62 mg; Se (Na₂SeO₃), 0.3 mg.

The positive control (PC) diet was supplemented with an ionophore coccidiostat (salinomycin, 60 mg/kg); the negative control (NC) diet did not contain any additive. The nisin diets were supplemented with increasing levels of the bacteriocin in liquid form (3.2, 9.7, 20.1 and 87.2 ml/kg, respectively). The concentrations of nisin in the final diets were as follows: 100, 300, 900 and 2700 IU nisin/g (group NI100, NI300, NI900 and NI2700, respectively).

Table 1. Composition of the basal diets and its calculated nutritive value.
Preparation of nisin and analysis of nisin concentrations

The nisin preparation was prepared according to technology elaborated at the Department of Biotechnology and Food Microbiology, Poznań University of Life Sciences, using the nisin-producing strain Lactococcus lactis subsp. lactis ATCC11454. The strain was grown into log phase in MRS broth, harvested and stored at -80°C in MRS amended with 20% (vol/vol) glycerol. The strain was propagated twice in MRS broth at 30°C before use (primary cultures). Primary cultivations were performed in batch systems using 5-liter fermenters (Bioflo III, New Brunswick). MRS medium without Tween 80 was inoculated with 2% (vol/vol) of an overnight culture of the L. lactis strain and incubated anaerobically (in nitrogen-flushed atmospheres) at 30°C for 16 h. The suspension was maintained at constant level of pH 6.0 by addition of 5M NaOH. The cells were separated from culture medium by membrane microfiltration. The filtrates were adjusted to pH 6.5 and treated with catalase (300 IU/mL, C-3515, Sigma) to exclude the antimicrobial effects of hydrogen peroxide and heated at 80°C for 10 min in order to inactivate proteases, catalase and kill any residual cells. The filtrate was further concentrated using ultrafiltration. The ultrafiltration process was carried out in an Amicon filtration system (model CH2RSA) equipped in cellulose acetate membranes with cut-off point of 30, and further of 5 kDa. The 10-fold concentrated fluid, containing over 800 mg/liter of nisin (32,000 IU/ml), was used as a feed additive in broiler chicken diets [14].

Nisin concentrations were analysed by a bioassay method based on the method of Matsuzaki et al. [15] as follows. Five millilitres of nutrient broth (Biocorp, Poland) was inoculated with Staphylococcus aureus ATCC 25923 and incubated on a shaker (100 strokes/min) at 30°C for 15 h. Fifty microlitres of the S. aureus cell suspension and 50 μL of the ultra-filterate of the nisin-producing L. lactis culture containing nisin solution were added to 5 ml of fresh medium, and this mixture was incubated under the same conditions. After 12 h (i.e. late log phase), the cell concentration was determined by measuring the OD600 using an UV spectrophotometer (model Spectord 205, JENA). The samples were diluted so that the OD600 was in range of 0.1 to 1.5 absorbance units. The OD600 values were observed to be inversely correlated with the amounts of nisin ultra-filterate added. A calibration curve was made using commercially available nisin standard (Sigma, 1000 IU/mg of solid). Nisin concentration was expressed in milligrams per litre, and a nisin concentration of 1 mg/litre was equivalent to 40 IU/ml [16].

Data and Sample Collection

The feed intake and body weight of the chickens were measured on days 14, 28 and 35. Mortality was registered throughout the entire experiment. At the end of the trial (35d) from each experimental group, 21 randomly picked chickens (3 chickens from 7 pens) were killed by cervical dislocation. For analyses of the gastrointestinal contents (bacterial enzymes, pH and organic acid concentrations), the contents of crop, ileum and caeca from 3 birds per pen were pooled (7 replicate digesta samples of approx. 10g). The remaining part of the samples was immediately frozen and stored in -80°C for the analysis of organic acids by gas chromatography and the microbiota composition by fluorescent in situ hybridization (FISH) of single bacterial cells.

Analysis of pH, Fermentation Products and Bacterial Enzyme Activities

The pH in the pooled digesta samples from crop, ileum and caeca, respectively, was measured immediately after slaughter using a combined glass and reference electrode.

Digesta samples were subjected to short-chain fatty acids (SCFA) analysis, using GC (Shimadzu GC-2010, Kyoto, Japan). The samples (0.5g crop and ileum samples, 0.2g caeca sample) were mixed with 0.2 ml formic acid, diluted with deionised water and centrifuged at 7,211 × g for 10 min. The supernatant was loaded onto a capillary column (SGE BP21, 30 m × 0.53 mm) using an on-column injector. The initial oven temperature was 85°C and was raised to 180°C by 8°C/min and held there for 3 min. The temperatures of the flame ionisation detector and the injection port were 180°C and 85°C, respectively. The sample volume used for GC analysis was 1 μl. The putrefactive SCFA (PSCFA) concentration was calculated as the sum of iso-butyrate, iso-valerate, and valerate concentration in the digesta.

The activity of bacterial enzymes (α- and β-glucosidase, α- and β-galactosidase and β-glucuronidase) in crop, ileum and caeca digesta (in the latter case additionally the activity of α-arabinopyranosidase and β-xylosidase were defined) was measured by the rate of p- or o-nitrophenol release from their nitrophenylglycosides, according to the method described elsewhere [17]. The following substrates were used: p-nitrophenyl-α-D-glucopyranoside (for α-glucosidase); p-nitrophenyl-β-D-glucopyranoside (for β-glucosidase); p-nitrophenyl-α-D-galactopyranoside (α-galactosidase); p-nitrophenyl-β-D-galactopyranoside (β-galactosidase); p-nitrophenyl-β-D-glucuronide (for β-glucuronidase); p-nitrophenyl-α-L-arabinopyranosidase (for α-arabinopyranosidase); p-nitrophenyl-β-D-xylopyranoside (for β-xylosidase). The reaction mixture contained 0.3 ml of a substrate solution (5 mM) and 0.2 mL of a 1:10 (v/v) dilution of the crop, ileum or caeca samples in 100mM phosphate buffer (pH 7.0) after centrifugation at 7211 g for 15 min. Incubation was carried out at 39°C and p-nitrophenol was quantified spectrophotometrically at A400 nm and at A395 nm (o-nitrophenol concentration) after the addition of 2.5 ml of 0.25 M-cold sodium carbonate. The enzymatic activity was expressed as μmol product formed per hour per g of digesta. The above outlined procedure determines the activities of extracellular bacterial enzymes released from bacterial cells into the digesta.

Microbial Community Analysis by Fluorescent In Situ Hybridization (FISH)

For FISH analysis, 100 μL of the ileum digesta were diluted in PBS and pipetted onto 0.22 μm polycarbonate filters (Frisenette K02BP02500) and vacuumed (Vacuum KNF Vacupoint-Neuberg). After vacuuming, the filters were transferred onto cellulose discs for dehydration in an ethanol.
series (50, 80, and 96%, 3 min. each). For each sample, a series of identical filters was prepared to allow the determination of optimal hybridization [18,19]. The oligonucleotides probes used for this study (Table 2) were selected from the literature. Hybridizations were carried out in 50 µL of hybridization buffer (0.9 M NaCl; 20 mM Tris/HCl, pH 7.2; 0.01% SDS) containing the oligonucleotides probes (Table 2). After hybridization, the filters were washed with washing buffer (20 mM Tris/HCl, pH 7.2; 0.01% SDS; 5 mM EDTA) for 20 min. at 48°C. The filters were rinsed gently in distilled water, air-dried, and mounted on object glasses with VectaShield (Vector laboratories nr. H-1000) anti fading agent containing DAPI (4',6-diamidino-2-phenylindole). To distinguish total count (DAPI) of bacteria from other particles in the ileum samples filters were left in 4°C for one hour in the dark until visualized using a Carl Zeiss Microscope Axio Imager M2.

**Statistical analysis**

The data were treated statistically using one-way analysis of variance, and significance of differences between groups was determined by the Duncan’s multiple range test at the significance level of $P$ equal to or less than 0.05. The calculations were tested using the GLM procedure of SAS software [20].

**Results**

**Microbial community analysis**

The total number of bacteria (DAPI counts) was lowest in the PC group (Table 3). Compared to the NC group, nisin did not affect the total bacterial counts. None of the dietary treatments affected the *Clostridium perfringens* and LAB counts. *Bacteroides* and *Enterobacteriaceae* counts, on the other hand, were influenced by all dietary treatments. The highest counts were observed in the NC group, whereas significant reductions were observed with salinomycin and nisin supplementation; for nisin in a dose-dependent manner.

**Microbial fermentation patterns and enzyme activities**

The microbial fermentation pattern in the broiler chicken GIT was affected by nisin and salinomycin in all examined segments (Figure 1-3, Table 4). In the crop, both additives significantly lowered the total SCFA level and dramatically changed the profile, particularly by reducing the level and proportion of propionate (Figure 1, Table 4). A similar trend was observed in ileum, however, statistically lower SCFA and PSCFA levels were observed only in the NI$_{100}$, NI$_{300}$ and NI$_{700}$ groups in comparison to the NC group (Table 4). Again, nisin reduced the proportion of propionate, whereas salinomycin did not exert this effect in ileum (Figure 2). Compared to crop and ileum, the highest total and individual SCFA and PSCFA levels were observed in the caeca digesta (Table 4). The lowest levels of acetic and propionic acids were detected with the

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**Table 2. Oligonucleotide probes.**

| Target                        | Probe   | Sequence (5' to 3')                      |
|-------------------------------|---------|-----------------------------------------|
| *Bacteroides* – *Prevotella* cluster | Bac303  | CCAATGTGGGGGGACCT$^f$                   |
| *Clostridium perfringens*     | Cperf191| GTAGTAAGTTGGTTCTCG2                     |
| *Enterobacteriaceae*          | Enter1432| CTTTGGCAACCACCT3                        |
| *Lactobacillus sp.* /*Enterococcus sp.* | Lab158  | GGTATTAGCRYCTTTCCA$^f$                  |

$^f$ [19$^{2-3}$, 50], $^*$[51]

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**Table 3. Selected microbial counts (log cfu/ml digesta) in ileal digesta determined by DAPI staining and fluorescent in situ hybridization (FISH).**

|                | PC       | NC       | NI$_{100}$ | NI$_{300}$ | NI$_{900}$ | NI$_{2700}$ | SEM   | $P$  |
|----------------|----------|----------|------------|------------|------------|------------|-------|------|
| DAPI           | 10.01$^b$| 10.04$^a$| 10.01$^a$  | 10.04$^a$  | 10.04$^a$  | 10.04$^a$  | 0.01  | <0.001|
| *Bacteroides*– *Prevotella* | 7.87$^c$ | 8.19$^a$ | 8.13$^b$   | 7.96$^c$   | 7.85$^a$   | 7.82$^a$   | 0.13  | <0.001|
| *Clostridium perfringens* | 7.77     | 7.71     | 7.87       | 7.82       | 7.82       | 7.70       | 0.13  | 0.369 |
| *Enterobacteriaceae*        | 8.01$^b$ | 8.27$^a$ | 7.93$^c$   | 7.76$^a$   | 7.97$^c$   | 7.96$^c$   | 0.16  | 0.002 |
| *Lactobacillus* /*Enterococcus* | 8.03     | 8.15     | 8.25       | 8.21       | 8.05       | 8.07       | 0.12  | 0.076 |

PC - positive control (salinomycin, 60 mg/kg); NC - negative control (any additives); NI$_{100}$ - 100 IU nisin/g (3.2 ml/kg); NI$_{300}$ - 300 IU nisin/g (9.7 ml/kg); NI$_{900}$ - 900 IU nisin/g (20.1 ml/kg); NI$_{2700}$ - 2700 IU nisin/g (87.2 ml/kg)

DAPI - total number of bacteria determined by 4',6-diamidino-2-phenylindole staining
SEM - standard error of the mean

Within the same row, different superscripts indicate significant differences between treatments ($P$ ≤ 0.05)

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highest dietary nisin levels. Iso-butyric acid was increased only by the salinomycin supplementation. Butyric acid concentration did not differ \((P<0.05)\) between PC, NI\(_{100}\), and NI\(_{2700}\) treatments. The highest concentration of iso-valeric acid was found in the PC group but it was only statistically different \((P<0.05)\) from the NI\(_{2700}\) group. Lowest valeric acid concentration was observed in NI\(_{2700}\) however only in case of NI\(_{300}\) it was statistically different \((P<0.05)\). The highest PSCFA were detected in PC while lowest in NI\(_{2700}\) \((P<0.05)\), lowest total SCFA in NI\(_{2700}\) \((P<0.05)\).

In the caeca, nisin and salinomycin supplementation did not affect the SCFA profile (Figure 3).

There was no effect of treatment on crop digesta pH (Table 5). Significant pH effects were observed in ileum and caeca digesta; the highest values were observed in the PC group and the lowest values in the NI groups, but without a clear dose-dependent pattern (Table 5).

In general, the dietary nisin and salinomycin supplementations significantly affected the activity of specific bacterial enzymes in crop, ileum and caeca (Table 6). In crop digesta, the activities of \(\alpha\)- and \(\beta\)-glucosidases, \(\alpha\)-galactosidases and \(\beta\)-glucuronidase, activities of \(\alpha\)-arabinopyranosidase and \(\beta\)-xylosidase were analysed as well. As compared to the NC group, nisin supplementation (NI\(_{900}\), NI\(_{300}\), NI\(_{2700}\)) significantly decreased the activity of \(\alpha\)- and \(\beta\)-glucosidases, \(\alpha\)-galactosidases \((\text{NI}_{2700})\), \(\beta\)-glucosidases \((\text{NI}_{2700})\), \(\beta\)-glucuronidase \((\text{NI}_{2700})\), \(\alpha\)-arabinopyranosidase \((\text{NI}_{2700})\); \(\beta\)-xylosidase \((\text{NI}_{2700})\).

**Bird performance**

In the 1-14 d period, the lowest body weight gain (BWG) was recorded in the PC group (Table 7). Nisin supplementation improved BWG in all treatments as compared to PC, however, birds fed the lowest dosage of the bacteriocin did not differ from the NC group \((P=0.001)\). In the 15-28 d period, only the NI\(_{900}\) and NI\(_{2700}\) groups were statistically different from the control groups. In the last period (29-35 d), the PC group was characterized by the highest BWG, but it was not statistically different from the NI\(_{900}\) and NI\(_{2700}\) groups. Over the entire experimental period (1-35 d), the highest BWG was observed for the nisin-supplemented NI\(_{900}\) and NI\(_{2700}\) groups. Dietary

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**Figure 1. SCFA profiles in crop digesta.** PC - positive control (salinomycin, 60 mg/kg); NC - negative control (any additives); NI\(_{100}\) - 100 IU nisin/g (3.2 ml/kg); NI\(_{300}\) - 300 IU nisin/g (9.7 ml/kg); NI\(_{900}\) - 900 IU nisin/g (20.1 ml/kg); NI\(_{2700}\) - 2700 IU nisin/g (87.2 ml/kg); Different letters indicate significant differences between treatments \((P \leq 0.05)\).

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additives also affected the feed conversion ratio (FCR). In the 1-14 d period, the lowest FCR were observed in the NI900 and NI2700 groups and highest in the PC group. These differences were not continued in later growth stages; in the 29-35 d period, salinomycin improved FCR to the same extent as NI2700 but numerically it was the lowest value among all treatments. In the entire experimental period (1-35 d), the lowest FCR was observed in the treatments with highest inclusion of nisin (NI900 and NI2700). Nisin and salinomycin effected feed intake (FI) of the broiler chickens. In the 1-14 d and 15-28 d periods, the highest FI was observed in the NI900 and NI2700 groups. A similar trend was observed over the entire experimental period (1-35 d).

Discussion

In general, the results of the present study demonstrate salinomycin as well as nisin to modulate the microbial ecology of the broiler GIT. The digesta concentrations of SCFAs were thus highest in the non-supplemented control group in each GIT segment, and the observed levels of total and individual SCFAs were in good agreement with our earlier studies [21,22]. Moreover, both additives changed the SCFA profiles of crop and ileum. The reduction of the fermentation activity in the GIT was generally in agreement with the observed bacterial enzyme activities. However, salinomycin and nisin showed slightly different modes of action. In ileum, α-glucosidase and β-galactosidase activities were highest in the salinomycin supplemented PC group, whereas nisin supplementation decreased these activities in a dose-dependent manner.

In the present study, none of the applied additives influenced pH in the crop, but significant pH effects were observed in the ileum and caeca. In contrast to our earlier studies with bacteriocins [2], the observed pH values did not reflect SCFA concentrations. Particularly in the crop, marked changes in total SCFA were observed without any concomitant effects on digesta pH (Table 5). The lack of consistency may relate to the fact that lactic acid, known to have a major influence on digesta pH, was not analyzed in the present study.

Nisin is reported to exert antimicrobial activity against numerous LAB and some bacteria belonging to the genera Staphylococcus, Micrococcus, Corynebacterium, Mycobacterium, Listeria, Clostridium and Bacillus [23,24]. In human studies it has been reported that nisin is inactivated by proteolytic enzymes and therefore exerts no effect on the GIT microbiota [10,25]. Data from in vivo and in vitro studies with ruminants, on the other hand, indicates that nisin can influence rumen fermentation and methanogenesis [26-28], and nisin has been reported to exert effects similar to the ionophore monensin [29]. These observations indicate that nisin is not immediately degraded in the rumen.

Figure 2. SCFA profiles in ileum digesta. PC - positive control (salinomycin, 60 mg/kg); NC- negative control (any additives); NI100 - 100 IU nisin/g (3.2 ml/kg); NI300 - 300 IU nisin/g (9.7 ml/kg); NI900 - 900 IU nisin/g (20.1 ml/kg); NI2700 - 2700 IU nisin/g (87.2 ml/kg); Different letters indicate significant differences between treatments (P≤ 0.05).

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The commercially available nisin products are dry and contain 2.5% nisin, 74.4% sodium chloride and 23.8% of denatured milk solids. This composition makes them unsuitable for poultry nutrition due to high amount of NaCl. However, in the available literature there are many other nisin preparations obtained from L. lactis fermentation, which can have relatively high nisin activity combined with low NaCl content. In poultry feeds, some additives (e.g. enzymes) are used in liquid form, thus development of the cheap method of nisin production would be very interesting from this point of view. However, it must be emphasized that even though nisin is used worldwide for food preservation purposes, it is not allowed to be used for animal nutrition in many countries. The FAO/WHO Codex Committee on milk and milk products accepted nisin as a food additive for processed cheese at a concentration of 12.5 mg pure nisin per kilogram product [30]. In some countries (e.g. Peru and France) nisin is accepted without any limits in particular foods (processed cheese) while in other countries there are strong national legislations (e.g. Argentina, USA, Italy) on maximum dosage. Besides food applications there is some work done on its clinical potential. In humans, there were successful attempts to use nisin in the treatment of atopic dermatitis [31], stomach ulcers and colon infections for patients with immune deficiencies [32], as well as staphylococcal mastitis during lactation in women. Bartoloni et al. [33] tested metronidazole, vancomycin, and nisin against 60 toxigenic strains of Clostridium difficile collected from human subjects with Clostridium difficile-associated diarrhea and observed nisin to much more effective than applied antibiotics. However, most studies agree that due to nisin susceptibility to degradation by the digestive enzymes, it cannot play any role in the development of the endogenous GIT microbiota [10,25].

The present study demonstrates that dietary nisin can modulate the microbial ecology of the broiler chicken GIT in a manner similar to the ionophore coccidiostat salinomycin. In contrast to our findings, work done on human microflora-associated rats indicated that dietary nisin had no effect on the gastrointestinal microbial ecology, whereas diet supplementation with nisin-producing Lactococcus lactis strain CHCC5826, significantly affected the microbial community [10]. The differences in the observations could be linked with differences in the digestion processes in mammals and birds [34-36]; particularly digesta passage time and, hence, nisin exposure to different proteolytic enzymes. In rats digesta passage time is rather slow and around 10-13h [37], whereas in broiler chickens passage time is very rapid and usually do not exceed 3-5h [36]. The duration of the experiments could have had an impact as well; the rats were given in two single dosages for two days whereas in the present study, nisin was fed to the birds throughout the experimental period of 35 d.

The growth promoting effect of salinomycin observed in the present study is in agreement with earlier observations on broiler chickens [38], ruminants [39] and pigs [40]. Many of the applied ionophore coccidiostats strongly influence the GIT

![Figure 3. SCFA profiles in caeca digesta.](image-url)
Table 4. SCFA concentrations in crop, ileum and caeca digesta (µmol/g digesta).

|          | PC   | NC   | NI100 | NI300 | NI900 | NI2700 | SEM  | P      |
|----------|------|------|-------|-------|-------|--------|------|--------|
| Crop     |      |      |       |       |       |        |      |        |
| acetic    | 7.34 | 9.54 | 7.27  | 7.56  | 6.92  | 6.56   | 0.39 | 0.055  |
| propionic | 0.02 | 2.51 | 0.04  | 0.04  | 0.04  | 0.07   | 0.17 | <0.001 |
| isobutyric| 0.16 | 0.16 | 0.10  | 0.16  | 0.15  | 0.15   | 0.02 | 0.361  |
| butyric   | 0.18 | 0.14 | 0.05  | 0.04  | 0.12  | 0.02   | 0.02 | 0.065  |
| iso-valeric| 0.01 | 0.05 | 0.00  | 0.00  | 0.02  | 0.01   | 0.01 | 0.097  |
| valeric   | 0.01 | 0.01 | 0.01  | 0.00  | 0.01  | 0.10   | 0.01 | 0.121  |
| PSCFA sum | 0.19 | 0.23 | 0.14  | 0.10  | 0.19  | 0.24   | 0.06 | 0.156  |
| total SCFA| 7.72 | 12.4 | 7.50  | 7.74  | 7.28  | 6.89   | 0.47 | <0.001 |

Ileum:
| acetic    | 4.61 | 4.39 | 4.11  | 4.04  | 3.80  | 4.16   | 0.17 | 0.263  |
| propionic | 0.38 | 0.33 | 0.04  | 0.05  | 0.05  | 0.06   | 0.04 | 0.001  |
| isobutyric| 0.38 | 1.38 | 1.03  | 0.25  | 0.42  | 0.21   | 0.10 | <0.001 |
| butyric   | 0.05 | 0.03 | 0.25  | 0.05  | 0.11  | 0.05   | 0.03 | 0.107  |
| iso-valeric| 0.02 | 0.00 | 0.05  | 0.02  | 0.04  | 0.04   | 0.01 | 0.339  |
| valeric   | 0.01 | 0.02 | 0.03  | 0.01  | 0.01  | 0.01   | 0.01 | 0.273  |
| PSCFA sum | 0.41 | 1.40 | 1.11  | 0.29  | 0.50  | 0.30   | 0.10 | <0.001 |
| total SCFA| 5.46 | 6.15 | 5.51  | 4.43  | 4.47  | 4.51   | 0.22 | 0.043  |

Caeca:
| acetic    | 53.2 | 61.0 | 59.1  | 59.3  | 57.2  | 48.0   | 1.32 | 0.006  |
| propionic | 4.38 | 4.09 | 4.25  | 4.10  | 3.82  | 2.86   | 0.17 | 0.016  |
| isobutyric| 0.69 | 0.41 | 0.44  | 0.40  | 0.45  | 0.38   | 0.03 | 0.012  |
| butyric   | 9.69 | 13.5 | 12.1  | 13.3  | 13.6  | 10.0   | 0.47 | 0.200  |
| iso-valeric| 0.48 | 0.29 | 0.38  | 0.36  | 0.42  | 0.21   | 0.03 | 0.010  |
| valeric   | 0.68 | 0.65 | 0.71  | 0.81  | 0.70  | 0.48   | 0.04 | 0.038  |
| PSCFA sum | 1.85 | 1.35 | 1.54  | 1.58  | 1.57  | 1.06   | 0.08 | 0.012  |
| total SCFA| 69.2 | 80.0 | 76.9  | 78.3  | 76.2  | 61.9   | 1.72 | 0.002  |

PC = positive control (salinomycin, 60 mg/kg); NC = negative control (any additives); NI100 = 100 IU nisin/g (3.2 ml/kg); NI300 = 300 IU nisin/g (9.7 ml/kg); NI900 = 900 IU nisin/g (20.1 ml/kg); NI2700 = 2700 IU nisin/g (87.2 ml/kg)

Table 5. pH in crop, ileum and caeca digesta.

|          | PC   | NC   | NI100 | NI300 | NI900 | NI2700 | SEM  | P      |
|----------|------|------|-------|-------|-------|--------|------|--------|
| pH       |      |      |       |       |       |        |      |        |
| Crop     | 4.95 | 4.82 | 4.90  | 4.94  | 4.93  | 0.03   | 0.714|
| ileum    | 5.84 | 5.72 | 5.53  | 5.53  | 5.37  | 0.05   | 0.032|
| Caeca    | 6.13 | 6.09 | 5.91  | 5.76  | 5.69  | 0.04   | <0.001|

PC = positive control (salinomycin, 60 mg/kg); NC = negative control (any additives); NI100 = 100 IU nisin/g (3.2 ml/kg); NI300 = 300 IU nisin/g (9.7 ml/kg); NI900 = 900 IU nisin/g (20.1 ml/kg); NI2700 = 2700 IU nisin/g (87.2 ml/kg)

As mentioned above, information on broiler chicken performance as influenced by the use of dietary bacteriocins is scarce [45]. In our previous work [2-5], we have demonstrated that dietary supplementation of the bacteriocin divercin AS7 in a liquid preparation improved broiler performance in a manner similar to the use of the ionophore coccidiatstat salinomycin. Likewise, pediocin A, produced by Pediococcus pentosaceus FBB61, was observed to improve growth and feed utilization of broiler chickens challenged with Clostridium perfringens [7]. A significant reduction of Campylobacter jejuni has also been demonstrated in turkey pouls after dietary addition of bacteriocin B602 from Paenibacillus polymyxa (NRRL B-30509) and bacteriocin OR7 from Lactobacillus salivarius NRRL B-35014 [46]. Finally,
### Table 6. Activity of extracellular bacterial enzymes in crop, ileum and caeca digesta (µmol/h/g digesta).

| Crop | PC | NC | Ni100 | Ni300 | Ni900 | Ni2700 | SEM | P      |
|------|----|----|-------|-------|-------|--------|-----|--------|
| α-glucosidase | 0.47<sup>b</sup> | 1.13<sup>a</sup> | 0.50<sup>b</sup> | 0.37<sup>b</sup> | 0.31<sup>b</sup> | 0.22<sup>b</sup> | 0.06<sup></sup> | <.0001 |
| β-glucosidase | 5.60<sup>b</sup> | 7.14<sup>a</sup> | 4.83<sup>bc</sup> | 4.45<sup>bc</sup> | 4.52<sup>bc</sup> | 3.94<sup>c</sup> | 0.23<sup></sup> | <.0001 |
| α-galactosidase | 14.9 | 16.5 | 16.1 | 15.6 | 16.2 | 14.7 | 0.43<sup></sup> | 0.313 |
| β-galactosidase | 0.99<sup>c</sup> | 6.33<sup>a</sup> | 4.84<sup>b</sup> | 4.18<sup>b</sup> | 4.21<sup>b</sup> | 2.26<sup>c</sup> | 0.34<sup></sup> | <.0001 |
| β-glucuronidase | 0.15<sup>ab</sup> | 0.18<sup>a</sup> | 0.13<sup>ab</sup> | 0.04<sup>b</sup> | 0.04<sup>b</sup> | 0.04<sup>b</sup> | 0.02<sup></sup> | 0.039 |

### Table 7. Broiler chicken performance.

| BWG (g/bird) | PC | NC | Ni100 | Ni300 | Ni900 | Ni2700 | SEM | P      |
|--------------|----|----|-------|-------|-------|--------|-----|--------|
| 1-14d | 313<sup>b</sup> | 332<sup>a</sup> | 332<sup>a</sup> | 346<sup>c</sup> | 360<sup>d</sup> | 381<sup>e</sup> | 3.12<sup></sup> | <.0001 |
| 14-28d | 859<sup>a</sup> | 858<sup>a</sup> | 899<sup>ab</sup> | 900<sup>ab</sup> | 932<sup>b</sup> | 946<sup>b</sup> | 8.16<sup></sup> | 0.003 |
| 28-35d | 591<sup>b</sup> | 539<sup>a</sup> | 520<sup>a</sup> | 530<sup>a</sup> | 556<sup>ab</sup> | 591<sup>b</sup> | 6.85<sup></sup> | 0.002 |
| 3-35d | 1763<sup>a</sup> | 1729<sup>a</sup> | 1751<sup>a</sup> | 1776<sup>a</sup> | 1847<sup>b</sup> | 1918<sup>c</sup> | 12.61<sup></sup> | <.0001 |
| FE (kg/kg) | 1-14d | 1.42<sup>d</sup> | 1.37<sup>a</sup> | 1.37<sup>a</sup> | 1.36<sup>a</sup> | 1.32<sup>c</sup> | 1.27<sup>b</sup> | 0.01<sup></sup> | <.0001 |
| 14-28d | 1.62 | 1.63 | 1.59 | 1.62 | 1.60 | 1.58 | 0.01<sup></sup> | 0.754 |
| 28-35d | 1.87<sup>b</sup> | 2.01<sup>c</sup> | 2.08<sup>a</sup> | 1.99<sup>ac</sup> | 1.97<sup>abc</sup> | 1.89<sup>bc</sup> | 0.02<sup></sup> | 0.001 |
| 3-35d | 1.66<sup>a</sup> | 1.70<sup>a</sup> | 1.69<sup>a</sup> | 1.68<sup>a</sup> | 1.65<sup>ab</sup> | 1.61<sup>b</sup> | 0.01<sup></sup> | 0.003 |
| FI (g/bird) | 1-14d | 446<sup>a</sup> | 456<sup>bc</sup> | 455<sup>a</sup> | 470<sup>bc</sup> | 475<sup>b</sup> | 483<sup>c</sup> | 2.50<sup></sup> | <.0001 |
| 14-28d | 1388<sup>a</sup> | 1391<sup>a</sup> | 1426<sup>bc</sup> | 1452<sup>bc</sup> | 1489<sup>b</sup> | 1493<sup>b</sup> | 8.54<sup></sup> | <.0001 |
| 28-35d | 1101 | 1079 | 1073 | 1052 | 1090 | 1113 | 7.79<sup></sup> | 0.281 |
| 3-35d | 2934<sup>a</sup> | 2926<sup>a</sup> | 2853<sup>ab</sup> | 2973<sup>ab</sup> | 3054<sup>bc</sup> | 3089<sup>f</sup> | 15.72<sup></sup> | 0.005 |

PC - positive control (salinomycin, 60 mg/kg); NC - negative control (any additives); Ni100 - 100 IU nisin/g (3.2 ml/kg); Ni300 - 300 IU nisin/g (9.7 ml/kg); Ni900 - 900 IU nisin/g (20.1 ml/kg); Ni2700 - 2700 IU nisin/g (87.2 ml/kg)

SEM - standard error of the mean

Within the same row, different superscripts indicate significant differences between treatments (P<0.05)

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dietary albusin B (bacteriocin) of Ruminococcus albus 7 expressed by yeast, has been reported to increase intestinal nutrient absorption, elevate the fecal Lactobacillus counts and decrease the population of Enterococcus and Salmonella, and thereby improving the growth performance of broiler chickens [47].

The results of the present work demonstrate that nisin exerted a clearly modulating effect on the microbial ecology of the GIT, and even in an unprotected form, the nisin ultra-filterate was able to improve bird performance significantly. Even though the detailed microbiological mechanisms behind the observed effects are still to be investigated for further elucidation, the present study is, to our knowledge, the first to demonstrate the dietary efficacy of this bacteriocin in broiler chickens. It is particular noteworthy that in comparison to salinomycin, dietary nisin in the NL100 and NL270 treatments, improved broiler chicken body weight gain by 4.7 and 8.7%, respectively (P<0.001). Moreover, birds from the NL270 group were characterized by the best feed utilization (P=0.003) among all groups. This phenomenon can be explained by several modes of action, as modulation of host immunity and stimulation of response to Eimeria, similar to ionophores [48]. Improved cellular immune response after Eimeria infection, resulting in reduced fecal oocyst shedding [49] or improved nutrient absorption and utilization [5]. Unfortunately, the present study did not include analysis of Eimeria sp. colonization of the broiler chickens, wherefore direct effects of the nisin on this parasite could not be evaluated.

Conclusions

The findings of the present study suggest that for broiler chickens, dietary nisin exerts a mode of action similar to that of the ionophore salinomycin; they both improve broiler growth performance by modulating the microbial ecology of the GIT. Dietary nisin supplementation may thus be considered a novel strategy in poultry production for improving broiler chicken feed utilization and growth performance presumably by reducing microbial density and activity in the GIT.

Author Contributions

Conceived and designed the experiments: DJ JJ. Performed the experiments: DJ BK MR AS. Analyzed the data: DJ BK JJ ZZ MR JD AS OH. Contributed reagents/materials/analysis tools: ZZ JJ DJ AS. Wrote the manuscript: DJ ZZ OH.

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Nisin in Broiler Chicken Diets

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