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Dietary divercin modifies gastrointestinal microbiota and improves growth performance in broiler chickens

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Running title: Divercin in broiler chicken diets

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Abstract 1. The aim of the present study was to investigate the effects of dietary administration of a divercin AS7 liquid preparation on broiler chicken performance, nutrient digestibility, counts of lactic acid bacteria (LAB) and coliform bacteria, as well as on the microbial activity in the gastrointestinal tract (GIT) as expressed by digesta pH and concentrations of short-chain fatty acids and lactic acid.

2. A total of 450 1-d-old male Ross 308 chickens were randomly distributed to three dietary treatments, with 15 pens per treatment and 10 birds per pen. The dietary treatments consisted of a positive control (PC) supplemented with 60 mg/kg salinomycin, a negative control (NC) without any additives, and the divercin (DIV) supplemented diet containing 0.2 ml/kg of the liquid divercin AS7 preparation.

3. The dietary divercin AS7 supplementation significantly increased body weight gain at 35 d compared to the NC group. Moreover, the pH of crop contents was higher and that of caecal contents lower in birds fed on the divercin supplemented diets.

4. Significantly lower counts of LAB were observed in the crops and caeca of the birds treated with divercin. Further, the divercin supplementation decreased lactic and succinic acid concentrations in the crop and ileum.

5. The present study demonstrates that the use of divercin supplemented diets can influence composition and activity of the microbiota in the broiler chicken GIT even in the lower parts that should otherwise not be targeted due to the peptide structure of the bacteriocin.

INTRODUCTION

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5 42 In recent years, a number of studies have demonstrated a close relationship between
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7 43 broiler performance, health and the gastrointestinal tract (GIT) microbiota (Engberg *et*
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9 44 *al.*, 2002; Maisonnier *et al.*, 2003; Engberg *et al.*, 2004; Choct 2009). Taking the
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11 45 complexity and biodiversity of the GIT micro-ecosystem into consideration, a
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13 46 manipulation of the GIT microbiota in a way that is profitable for the host-bird and for
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15 47 the farmer, however, seems a difficult goal to achieve. Several studies have
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17 48 demonstrated that the protective potential of the native commensal microbiota may
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19 49 easily be disrupted by different nutritional factors (Dahiya *et al.* 2006; Choct 2009),
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21 50 thereby compromising one of the suggested ways for the commensal microbiota to
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23 51 support performance and health of broilers; namely elimination of pathogenic bacteria
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25 52 from the GIT. On the other hand, it is also well recognised that the commensal
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27 53 microbiota, no matter how protective it may be, does impose a burden upon the host
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29 54 that goes beyond simply competing for feed components (Engberg *et al.*, 2000), *via*
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31 55 more specific mechanisms such as compromising fat digestibility (Knarreborg *et al.*
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33 56 2002). A mere reduction in the commensal microbial load of the GIT may therefore, in
34
35 57 a short term perspective, represent a growth promoting factor for the host, though
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37 58 admittedly, it may at the same time render the host more susceptible to pathogen
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39 59 invasion.

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47 60 Probiotics with potentially beneficial effects on broiler growth and health have
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49 61 gained considerable interest (Choct 2009) and have been shown to stimulate the
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51 62 immune system and to reduce the growth and intestinal colonisation of pathogenic
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53 63 bacteria by competitive exclusion (Rijkers *et al.* 2010). Further, some probiotic
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64 bacteria produce different types of peptides, *e.g.* bacteriocins, with direct antimicrobial
65 effects (Joerger 2003). Even though bacteriocins have been used in human nutrition for
66 decades (Cleveland *et al.*, 2001; Joerger 2003; Leisner *et al.*, 2007), only limited
67 information is available regarding the effects of directly fed bacteriocins on broiler
68 chickens. A few recent publications do however indicate that different bacteriocins
69 administered with the feed may inhibit GIT colonisation by *Campylobacter jejuni* and
70 *Salmonella enteritidis* (Stern *et al.*, 2005; Line *et al.*, 2008).

71 Among bacteria capable of producing bacteriocins, much attention has been
72 paid to the lactic acid bacteria, LAB (Stern *et al.*, 2006; Svetoch *et al.*, 2008).
73 *Carnobacterium* is a genus of LAB that can be isolated from various sources such as
74 the GIT of fish, dairy products and alpine permafrost (Leisner *et al.*, 2007). The
75 *Carnobacterium* genus comprises nine described species, of which *Carnobacterium*
76 *divergens* and *Carnobacterium maltaromaticum* have been most widely studied, due to
77 their role in the inhibition of many pathogenic and food spoilage bacteria such as
78 *Clostridium*, *Bacillus*, *Brochothrix*, *Streptococcus*, *Staphylococcus* and *Micrococcus*.
79 They do, however, also possess antimicrobial activity against fellow LAB of the genera
80 *Lactobacillus*, *Leuconostoc*, *Carnobacterium*, *Pediococcus* and *Enterococcus* (Sip *et*
81 *al.*, 1998; Leisner *et al.*, 2007); a feature potentially related to *Carnobacterium*
82 bacteriocin production.

83 *Carnobacterium divergens* produces the subclass IIa bacteriocins consisting of
84 30 to 60 amino acids (Leisner *et al.* 2007) and so far, three major bacteriocins produced
85 by this species have been identified; divercin V41 (Rihakova *et al.* 2009), divercin AS7

(Sip *et al.* 1998) and divergicin M35 (Tahiri *et al.* 2004). Apart from the data from our preliminary study (Józefiak *et al.*, 2010), no information is so far available on the use of bacteriocins produced by *Carnobacterium divergens* as feed additives for broiler chickens. The aim of the present study was therefore to investigate the effects of a divercin AS7 preparation on broiler chicken performance, nutrient digestibility, counts of coliform bacteria (indicator of potential pathogens) and LAB (representative commensals) as well as on the microbial activity in the GIT expressed by the levels of pH and short-chain fatty acids.

MATERIALS AND METHODS

Birds and housing

The effect of the divercin AS7 supplementation was evaluated in a growth performance experiment with broiler chickens kept in floor pens (1.58 × 1.16 m) over an experimental period of 35 d. A total of 450 1-d-old male ROSS 308 chicks were randomly distributed to three dietary treatments using 15 replicate pens per treatment and 10 birds per pen. The birds were given 23 h light and 1 h dark during the first week, and then 19 h light and 5 h dark from d 7 to 21. From 22 to 42 d of age, there was 23 h light and 1 h dark. The positive control (PC) diet was supplemented with an ionophore coccidiostat (salinomycin, 60 mg/kg), the negative control diet (NC) did not contain any additive and the divercin diet (DIV) was supplemented with 200 AU divercin/ml (0.2ml/ kg).

Diets and feeding programme

The compositions of the experimental diets are presented in Table 1. The divercin

activity was quantified by two-fold dilutions, and expressed in activity units (AU) per millilitre as previously described by Ennahar (2001). The divercin AS7 preparation was produced according to the technology elaborated by Sip *et al.* (1999) and was provided in liquid form. In the last 5 d of the experiment, 0.2% of the wheat was replaced by titanium oxide as an internal marker for digestibility analyses.

Table 1 near here

Data collection

The feed intake and body weight of the chickens were measured on d 14 and 35. Mortality was registered throughout the entire experiment. At the end of the trial (35 d) from each experimental group, 21 randomly picked chickens (three chickens from 7 pens) were killed by cervical dislocation. For analyses of gastrointestinal contents (enumeration of bacteria, pH and organic acid concentrations), the contents of crop, ileum and caecum from three birds per pen were pooled (7 replicate digesta samples of approximately 10 g). Approximately 5 g of the sample were used freshly for the culturing of intestinal bacteria and for pH analyses. The rest of the samples were immediately frozen and stored at -20°C for the analysis of organic acids by gas chromatography.

For the evaluation of apparent metabolisable energy (AME_N) and nutrient digestibility, a further 15 randomly chosen birds per treatment (one bird per pen) were killed on d 35 and ileal contents were collected. The ileum was removed and digesta were flushed from the terminal ileum (15 cm adjacent to the ileo-caecal junction). To provide sufficient material for chemical analysis, the fresh ileal samples were pooled into 5 replicates of approximately 60 g. From the same pens excreta samples of approx.

100 g were collected at 35 d. The digesta and excreta samples were immediately frozen, freeze-dried and ground before further analyses.

Chemical analyses

Feed samples were analysed in duplicate for crude protein, crude fat, and crude fibre using AOAC (2005) methods 976.05, 920.39 and 2002.04, respectively. For all chemical analysis, samples were ground to pass through a 0.5 mm sieve. Titanium dioxide was determined according to the method described by Short *et al.* (1996), and samples were prepared according to the procedure proposed by Myers *et al.* (2004). Gross energy was determined using an adiabatic bomb calorimeter (KL 12Mn, Precyzja-Bit PPHU, Poland) standardised with benzoic acid. Nitrogen content was analysed by the Kjel Foss Automatic 16210 (A/S N. Foss Electric, Denmark) and the crude protein content was calculated using a multiplication factor of 6.25. Fat content was determined using Soxhlet System HT 1043, Extraction Unit (Foss Tecator Denmark).

Microbiological analyses

The pH of pooled digesta from the crop, ileum and caecum was measured immediately after slaughter using a combined glass and reference electrode. The rest of the sample (2.5 g), was diluted in 30 ml of sterile pre-reduced salt medium (Miller and Wolin 1974). For enumeration of bacteria, the suspension was homogenised for two minutes in CO₂-flushed plastic bags using a stomacher homogeniser (Interscience, France). Subsequently, the samples were serially diluted in 10-fold steps using pre-reduced salt medium according to the technique of Miller and Wolin (1974). Lactic acid bacteria

were enumerated by spread-plating on de Man, Rogosa and Sharp (MRS) agar (Merck 1.10660) incubated for 48 h at 38°C in anaerobic jars (Anaerocult® A, Merck 1.13829). Coliform bacteria were enumerated by spread-plating on MacConkey agar (Merck 1.05465) incubated aerobically for 24 h at 38°C.

The concentration of short-chain fatty acids and lactic acid in the contents of the different gastrointestinal segments was determined by gas chromatography as described earlier (Canibe *et al.*, 2007) using a Hewlett Packard gas chromatograph (Model 6890, Hewlett Packard, Agilent Technologies, Naerum, Denmark) equipped with a flame-ionisation detector and a 30-m ZB-5 column with an internal diameter of 0.32 mm and coated with 5%-phenyl 95%-dimethylpolysiloxane with a film thickness of 0.25 µm. Total concentration of organic acids is expressed as the sum of acetic, propionic, butyric, isobutyric, valeric, succinic and lactic acids.

Digestibility calculations

The following equations were used to calculate the apparent digestibility of the protein, fat (using titanium dioxide and fat digestibility (dig.) calculation as an example) and AME_N content of experimental diets:

$$Fat\ dig.\ (\%) = \{1 - [(TiO_2\%_{diet}/TiO_2\%_{digesta/excreta}) \times (fat\%_{digesta/excreta}/fat\%_{diet})]\} \times 100$$

$$AME_N\ (kJ/kg) = GE_{kJ/kg\ diet} - [GE_{kJ/kg\ excreta} \cdot (TiO_2\%_{diet}/TiO_2\%_{excreta})] - 34.39 \cdot \{N\%_{diet} - [N\%_{excreta} \cdot (TiO_2\%_{diet}/TiO_2\%_{excreta})]\}$$

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7 175 Where GE is gross energy, N is nitrogen, TiO_2 is titanium oxide, and 34.39 is the

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10 176 energy equivalent of uric acid nitrogen (i.e., 34.39 kJ/kg uric acid nitrogen).

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12 177 **Statistical analysis**

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14 178 The study was set up as a completely randomised design, and data were tested using

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16 179 the GLM procedure of SAS (SAS 1990). Means were separated using a Duncan's

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18 180 multiple range test. All statements of significance are based on $P < 0.05$.

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21 181 **RESULTS**

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23 182 Throughout the trial, the mortality was below 2% without any signs of viral or bacterial

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25 183 infections. Birds fed on diets supplemented with salinomycin showed the best

26
27 184 performance results (Table 2). Compared with the non-supplemented control, the

28
29 185 divercin supplementation increased the body weight gain in the starter period

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31 186 ($P = 0.006$) and at the end of the trial ($P < 0.001$), and tended to improve total feed

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33 187 conversion ratio as well as feed intake in each growth stage.

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Tables 2 & 3 near here

188 The levels of apparent metabolisable energy (AME_N) and ileal digestible

189 energy (Table 3) increased after salinomycin supplementation ($P < 0.001$ and $P = 0.016$,

190 respectively). However, there was no difference between PC and DIV treatments with

191 respect to the estimated ileal digestible energy. Although the ileal crude fat digestibility

192 of birds fed the PC diet was approximately 24 percentage points higher than that of the

193 other groups (0.92 vs. 0.68), no statistical significant difference was found ($P = 0.118$).

194 Total tract fat digestibility was significantly higher ($P = 0.013$) in the PC group (Table

195 3). The lowest ileal crude protein digestibility, as well as total nitrogen retention, was
196 observed in the birds fed the NC diet.

Table 4 near here

197 In the present trial, the dietary supplementation with salinomycin numerically
198 increased the counts of LAB bacteria (Table 4), but only significantly in the caecum
199 ($P=0.015$). Divercin tended to reduce the LAB population in the contents of the crop
200 ($P=0.14$). However in the ileum ($P=0.015$) and caecum ($P=0.006$) the reduction was
201 significant. The counts of coliform bacteria in the different intestinal segments were
202 not influenced by the dietary treatments ($P>0.05$).

203 Compared with the non-supplemented controls, the digesta pH in crop and
204 ileum was higher when birds were fed salinomycin and divercin ($P=0.011$ and
205 $P=0.001$, respectively). In the caecum, the pH was highest in the non-supplemented
206 control ($P=0.043$).

Table 5 near here

207 In crop contents, the dominant organic acids were lactic acid and acetic acid
208 (Table 5), while succinic acid and butyric acid were detected in small concentrations.
209 Compared with the non-supplemented diet, the addition of salinomycin and divercin
210 reduced lactic acid, acetic acid and succinic acid concentrations by approximately 50%
211 ($P>0.001$) in crop contents. Also in ileal content, the most abundant organic acids were
212 lactic and acetic acid, whereas succinic acid was detected only in low concentrations
213 (Table 5). The concentration of lactic acid was influenced by the dietary treatment
214 ($P<0.001$) and was lowest in ileal contents of birds supplemented with salinomycin
215 ($34.5 \mu\text{g/g}$), as compared with divercin ($72.4 \mu\text{g/g}$) and the non-supplemented control
216 diet ($114.0 \mu\text{g/g}$). The concentration of acetic acid in ileal contents was not influenced

217 by the dietary treatment ($P>0.05$). In caecal contents, the organic acid profile was
218 much more diverse with acetic acid, butyric acid and propionic acid being the
219 dominant organic acids (Table 5). Low concentrations of isobutyric, isovaleric and
220 valeric acids were detected in caecal contents, whereas lactic acid was not detected at
221 this location. In general, divercin reduced the microbial fermentation to the same extent
222 as salinomycin (Table 5). There was no difference between groups with respect to the
223 production of organic acids in the caecum ($P>0.05$).

224 DISCUSSION

225 The observed growth promoting effect of salinomycin is in good agreement with
226 earlier studies on broiler chickens (Engberg *et al.*, 2000), ruminants (Spears 1990) and
227 pigs (Moore *et al.*, 1986). In poultry, many of the applied ionophore coccidiostats do
228 not only target *Eimeria* species but also certain Gram-positive GIT bacteria. These
229 substances have been observed to reduce the numbers of, for example, *Clostridium*
230 *perfringens* (Bjerrum *et al.*, 2005) and LAB, including species like *Lactobacillus*
231 *salivarius*, previously reported to be dominating in the broiler GIT (Engberg *et al.*,
232 2000). The improvement of bird growth and feed utilisation imposed by salinomycin
233 may be explained by a limitation of microbial growth and activity *per se*, alleviating
234 the direct competition with the host for nutrients. More specifically, by targeting
235 bacteria responsible for deconjugation of bile salts (Knarreborg *et al.* 2002; Guban *et*
236 *al.* 2006), salinomycin treatment may also improve fat digestibility (Knarreborg *et al.*
237 2004). In accordance with this, the present study demonstrated an increased fat
238 digestibility and AME_N content after dietary salinomycin supplementation. The AME_N

content was actually higher than the calculated energy value of the diets (13.6 vs. 13.0 MJ/kg). Compared with the NC diet, the ileal digestible energy increased after divercin supplementation (10.3 vs. 11.6 MJ/kg), being in between the NC and the PC diet. Both additives also improved ileal crude protein digestibility as well as nitrogen retention. The present results thus suggest that the divercin-medicated improvement in broiler growth performance ($P=0.001$) and feed utilisation ($P=0.059$) may be directly linked to an improved nutrient digestibility, due to a reduction of the GIT microbial load and activity..

Dietary supplementation with different bacteriocins has been shown to suppress different pathogenic bacteria. Cole *et al.* (2006) demonstrated significant reduction of *Campylobacter jejuni* in turkey poultlets after dietary addition of bacteriocin B602 from *Paenibacillus polymyxa* (NRRL B-30509), and bacteriocin OR7 from *Lactobacillus salivarius* (NRRL B-35014). *Enterococcus faecium* strain J96 isolated from the crop of a chicken showed protective effects on chickens infected with *Salmonella pullorum* (Audisio *et al.*, 1999). Mountzouris *et al.* (2010) examined avilamicin and a probiotic product based on 5 bacterial species isolated from the crop (*Lactobacillus reuteri* DSM 16350), jejunum (*Enterococcus faecium* DSM 16211), ileum (*Bifidobacterium animalis* DSM 16284), and caecum (*Pediococcus acidilactici* DSM 16210, and *Lactobacillus salivarius* DSM 16351) of healthy adult chickens. These bacteria are all capable of producing bacteriocins (Cleveland *et al.*, 2001; Joerger 2003; Diez-Gonzalez, 2007; Nazef *et al.*, 2008), and it has thus been concluded that some of the growth promoting effects were linked to antimicrobial activity of those peptides.

261 However, the highest ileal apparent digestibility coefficients of crude protein and ether
262 extract were observed in avilamycin supplemented treatment, followed by the
263 probiotic-supplemented group (Mountzouris *et al.*, 2010).

264 Grilli *et al.*, (2009) showed that pediocin A from *Pediococcus pentosaceus*
265 FBB61, belonging to the class III bacteriocins, significantly improved growth
266 performance of broiler chickens challenged with *Clostridium perfringens*. In the
267 present study, we did not enumerate specific pathogens. However, the experimental
268 diets provided animal fat sources (lard and tallow) known to stimulate the intestinal
269 growth of *Clostridium perfringens* (Knarreborg *et al.*, 2004). It cannot be excluded
270 therefore, that some of the observed salinomycin and divercin effects may be linked to
271 a suppression of *C. perfringens*; a very active player in bile acid de-conjugation
272 (Knarreborg *et al.*, 2002).

273 Lactobacilli are among the most abundant bacteria in the chicken GIT and
274 typically dominate in the upper GIT segments (crop, gizzard and ileum). Lu *et al.*
275 (2003) thus reported approx. 70% of the sequences in an ileum-derived 16S rRNA
276 gene library to be allocated to the *Lactobacillus* genus. In the caecum, on the other
277 hand, where strict anaerobes proliferate readily, lactobacilli only accounted for approx.
278 8% of the library sequences (Lu *et al.*, 2003). Lactic acid bacteria are usually
279 considered beneficial for the host bird due to their production of, for example, lactic
280 and acetic acids, leading to pH reduction (Engberg *et al.*, 2004), as well as their
281 production of certain bacteriocins (Nazef *et al.*, 2008). On the other hand, there is also
282 certain evidence that some small intestine LAB, *e.g.* *Lactobacillus salivarius* and

283 *Enterococcus faecium* might have negative effects on broiler performance (Engberg *et*
284 *al.* 2000; Guban *et al.* 2006).

285 In general, the observations of the present study suggest salinomycin as well as
286 divercin can suppress the overall microbial activity of the broiler GIT; the
287 concentration of organic acids was thus highest in the non-supplemented control group
288 in each GIT segment (Table 5), though not significant for the caecum, and the observed
289 levels of total and individual organic acids were in good agreement with our earlier
290 studies (Józefiak *et al.*, 2006; Józefiak *et al.*, 2007). The suppression of the microbial
291 activity by salinomycin and divercin is clearly reflected in the pH levels of the upper
292 GIT segments, but not in the caecum; an apparent discrepancy also observed earlier
293 (Engberg *et al.*, 2000). More specifically, the LAB activity also seemed to be
294 suppressed by salinomycin and divercin, as indicated by the lower lactic acid
295 concentrations and higher pH in the upper parts of the GIT (crop and ileum), compared
296 with the control group. Surprisingly, however, significant reduction of the LAB (MRS)
297 counts was only observed in the ileum of the divercin treated broilers, whereas
298 salinomycin actually increased caecal LAB counts; a discrepancy that cannot be readily
299 explained, but similar trends have been observed before (Engberg *et al.*, 2000) and it
300 emphasises the complexity of the mechanisms involved. In particular, the question of
301 which species of broiler GIT lactic acid bacteria may be targeted by divercin and
302 salinomycin remains unanswered, as no detailed identification of the involved LAB has
303 been conducted.

304 A recent study by Shin *et al.* (2008) showed that the broiler chicken GIT is inhabited

305 by many bacteriocin-producing bacteria with antibacterial activity against pathogens.
306 Even though *Carnobacteria* are relatively widespread in nature, however, there is so far
307 no evidence that they are present in the GIT of broiler chickens (Leisner *et al.*, 2007); a
308 fact that may explain the strong effects of divercin AS7 introduction on bacterial
309 fermentative activity. The fact that divercin AS7 in the present study tended to
310 suppress LAB even in the caecum indicates that this bacteriocin, even in an
311 unprotected form, may continue to be active in the lower segments of the broiler GIT,
312 although, as a protein, it is prone to hydrolysis in the upper intestine.

313 To our knowledge, the present study is the first attempt to use a
314 *Carnobacterium divergens* bacteriocin as a feed supplement and to demonstrate
315 positive growth promoting and antibacterial effects of divercin in broiler chickens.
316 Although consistent with our preliminary studies (Józefiak *et al.* 2010), the results
317 presented here should be followed up by further investigations in order to provide
318 more detailed information on broiler growth performance, for example, in response to
319 different divercin doses; as well as the specific bacteria being targeted by the
320 bacteriocin. Finally, it could be attempted to increase the effectiveness of divercin in
321 the broiler GIT by protective encapsulation of the peptide.

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469 **Table 1.** *Composition of the basal diets and their calculated nutritive value*

Ingredients (g kg^{-1})	Diet (0 – 35 d)
Wheat	326.8
Barley	250.0
Soyabean 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 ^a	5.0
Limestone	4.2
L-Lysine -HCl	2.8
DL-Methionine	2.1
L-Threonine	0.3
Sodium carbonate (Na_2CO_3)	1.0
Salt (NaCl)	2.6
Titanium oxide (TiO_2) ^b	2.0
<i>Calculated nutritive value (g kg^{-1})</i>	
ME (MJ kg^{-1})	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
<i>Analysed composition (g kg^{-1})</i>	
Crude protein	214.0
Crude fibre	39.2
Crude fat	101.2
ME (MJ kg^{-1})	12.89

470 ^aProviding the following per kilogram of diet: vitamin A (retinol), 11.166 IU; cholecalciferol, 2.500 IU;
 471 vitamin E (alpha tocopherol), 80 mg; menadione, 2.50 mg; cobalamin, 0.02 mg; folic acid, 1.17 mg;
 472 choline, 379 mg; D-pantothenic acid, 12.50 mg; riboflavin, 7.0 mg; niacin, 41.67 mg; thiamin, 2.17 mg;
 473 D-biotin, 0.18 mg; pyridoxine, 4.0 mg; ethoxyquin, 0.09 mg; Mn (MnO_2), 73 mg; Zn (ZnO), 55 mg; Fe
 474 (FeSO_4), 45 mg; Cu (CuSO_4), 20 mg; I (CaI_2O_6), 0.62 mg; Se (Na_2SeO_3), 0.3 mg.

475 ^bReplaced corresponding amount of the wheat in each diet, from 30-35 d of broiler growth

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Table 2. Performance results

Item	Treatment			SEM ^d	<i>P</i> ^e
	PC ^a	NC ^b	DIV ^c		
	Feed conversion ratio (g feed/g gain)				
1 – 14 d	1.48 ^y	1.54 ^x	1.49 ^y	0.01	0.001
14 – 35 d	1.60	1.67	1.67	0.02	0.140
1 – 35 d	1.58	1.65	1.61	0.01	0.059
	Body weight gain (g/bird)				
1 – 14 d	328 ^x	304 ^y	320 ^x	3.37	0.006
14 – 35 d	1474 ^x	1360 ^y	1413 ^{xy}	14.25	0.003
1 – 35 d	1801 ^x	1664 ^z	1733 ^y	15.83	<0.001
	Feed intake (g/bird)				
1 – 14 d	484	469	476	3.77	0.236
14 – 35 d	2357	2274	2349	18.99	0.138
1 – 35 d	2838	2737	2790	18.21	0.074

478 ^aThe positive control (PC) diet contained 60 mg/kg of salinomycin
479 ^bThe negative control (NC) diet did not contain any additives
480 ^cThe divercin supplemented diet contained 200 AU of divercin/ml (0.2 ml/kg)
481 ^dSEM – standard error of the mean
482 ^eP – probability
483 ^{x, y, z} Within rows, mean values without a common superscript differ significantly.

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490 **Table 3.** *Ileal digestibility of nutrients and energy, nitrogen retention and AME_N*491 *content.*

Item	Treatment			SEM ^d	P ^e
	PC ^a	NC ^b	DIV ^c		
	(kcal)				
AMEn	13.6 ^x	12.1 ^y	12.1 ^y	0.211	<0.001
Ileal digestible energy	12.9 ^x	10.3 ^y	11.6 ^{xy}	0.440	0.016
Ileal crude protein digestibility	0.74	0.62	0.70	0.021	0.074
N Retention	0.49 ^x	0.37 ^y	0.40 ^{xy}	0.021	0.048
Ileal crude fat digestibility	0.92	0.67	0.69	0.055	0.118
Total tract fat digestibility	0.90 ^x	0.76 ^y	0.72 ^x	0.028	0.013

492 ^aThe positive control (PC) diet contained 60 mg/kg of salinomycin493 ^bThe negative control (NC) diet did not contain any additives494 ^cThe divercin supplemented diet contained 200 AU of divercin/ml (0.2 ml/kg)495 ^dSEM – standard error of the mean496 ^eP – probability497 ^{x, y, z} Within rows, mean values without a common superscript differ significantly.

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Table 4. *Counts of coliform and lactic acid bacteria (log cfu/g digesta) and pH in crop, ileal and caecal digesta*

Item	Treatment			SEM ^d	<i>P</i> ^e
	PC ^a	NC ^b	DIV ^c		
	Crop				
Coliforms	6.98	7.08	6.55	0.110	0.112
Lactic acid bacteria	7.92	7.93	7.41	0.124	0.140
pH	4.33 ^x	3.94 ^y	4.31 ^x	0.189	0.011
	Ileum				
Coliforms	6.47	6.29	6.24	0.144	0.785
Lactic acid bacteria	7.90 ^y	7.72 ^y	7.03 ^x	0.142	0.015
pH	6.28 ^x	5.14 ^y	5.55 ^x	0.376	0.001
	Caecum				
Coliforms	7.88	7.71	7.13	0.191	0.253
Lactic acid bacteria	9.24 ^x	8.53 ^y	8.00 ^y	0.180	0.006
pH	5.49 ^y	5.78 ^x	5.52 ^y	0.181	0.043

^aThe positive control (PC) diet contained 60 mg/kg of salinomycin
^bThe negative control (NC) diet did not contain any additives
^cThe divercin supplemented diet contained 200 AU of divercin/ml (0.2 ml/kg)
^dSEM – standard error of the mean
^eP – probability
^{x, y, z} Within rows, mean values without a common superscript differ significantly.

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Table 5. Concentrations of organic acids ($\mu\text{mol/g}$ digesta) in the contents of crop, ileum and caecum of broiler chickens.

Item	Treatment			SEM ^d	P ^e
	PC ^a	NC ^b	DIV ^c		
	Crop				
Acetic acid	11.3 ^y	26.8 ^x	11.4 ^y	2.23	<0.001
Butyric acid	0.2	0.7	0.7	0.16	0.456
Lactic acid	55.3 ^y	119.0 ^x	52.0 ^y	9.59	<0.001
Succinic acid	2.8 ^y	8.7 ^x	3.2 ^y	0.79	<0.001
Total organic acids	69.7 ^y	155.0 ^x	67.3 ^y	12.36	<0.001
	Ileum				
Acetic acid	5.8	6.1	4.2	0.49	0.241
Lactic acid	34.5 ^z	114.0 ^x	72.4 ^y	10.37	<0.001
Succinic acid	0.2 ^y	2.0 ^x	0.7 ^y	0.27	0.006
Total organic acids	40.7 ^z	122.0 ^x	77.2 ^y	10.69	0.001
	Ceacum				
Acetic acid	86.5	92.0	79.6	3.34	0.32
Propionic acid	18.4	16.8	18.4	1.02	0.78
Isobutyric acid	0.9	1.2	1.0	0.10	0.49
Butyric acid	19.8	23.1	19.4	1.44	0.53
Isovaleric acid	0.5	0.6	0.5	0.08	0.95
Valeric acid	3.4	2.9	2.7	0.14	0.20
Succinic acid	ND ^f	4.35	1.32	-	-
Total organic acids	129.5	141.0	123.0	4.87	0.31

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^aThe positive control (PC) diet contained 60 mg/kg of salinomycin

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^bThe negative control (NC) diet did not contain any additives

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^cThe divercin supplemented diet contained 200 AU of divercin/ml (0.2mg/kg)

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^dSEM – standard error of the mean

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^eP – probability

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^fND – not detected

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^{x, y, z} Within rows, mean values without a common superscript differ significantly.

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