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The effects of *Carnobacterium divergens* AS7 bacteriocin on gastrointestinal microflora *in vitro* and on nutrient retention in broiler chickens

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ABSTRACT

The present study was undertaken to evaluate the effects of divercin, a bacteriocin produced by lactobacilli strain *Carnobacterium divergens* AS7, on the microflora status under *in vitro* conditions and on nutrient retention and nitrogen-corrected apparent metabolizable energy (AME_N) of divercin in an *in vivo* trial on broiler chickens. Low (DL) 200 AU·ml^1 (0.05% of the liquid divercin prepatation), and high (DH) 1600 AU·ml^{-1}(0.4% of the liquid divercin prepatation) doses of divercin were used in both trials. In the *in vitro* trial divercin at concentration, 1600AU ml^{-1} of divercin had stronger antibacterial effects as compared with 200 AU·ml^{-1}. In the crop and ileal digesta, the DH treatment was characterized by the lowest lactic acid bacteria (LAB) and coliform bacteria counts (0.4-0.8 log cycle reduction). There were no differences in nutrient retention between treatments. Salinomycin and divercin supplementation tended to increase fat digestibility and N retention, however. The highest AME_N were obtained in the DL treatment. The results of both studies show positive effects of divercin in terms of reduction of microbial populations isolated from the gastrointestinal tract (GIT) of broiler chickens as well as improvement in AME_N. The presented data may suggest that bacteriocin derived from *Carnobacterium divergens* AS7 could play a role in controlling the microbial ecosystem in the broiler chicken GIT.

KEY WORDS: broiler chickens, divercin, intestinal microflora, digestibility

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INTRODUCTION

One of the major issues of modern poultry nutrition is maintaining microflora homeostasis and gut health of the birds through dietary factors (Mead, 2000; Guarner and Malagelada, 2003; Joerger, 2003). In the post-antibiotic-growth-promoters era, many strategies have been proposed to eliminate pathogenic microbiota from the poultry gastrointestinal tract (GIT). This includes probiotics, prebiotics, organic acids, essential oils, etc. (Ricke, 2003; Józefiak et al., 2007). Little is known, however, about bacteriocins - extracellularly released proteinaceous compounds lethal to bacteria other than the producing strain (Joerger, 2003; Sip and Grajek, 2005). They display a limited antibacterial spectrum encompassing closely related strains in particular. Bacteriocins are gaining interest because of their wide antibacterial spectrum with feasible application in feeds and foods for controlling spoilage and pathogenic microorganisms (Joerger, 2003; Sip and Grajek, 2005; Stern et al., 2006; Rihakova et al., 2009). Many bacteriocins are produced by all genera of lactic acid bacteria (LAB) and they are generally recognized as safe (GRAS). One of the interesting bacteriocins is divercin AS7 produced by an atypical lactobacilli strain, *Carnobacterium divergens* AS7 (Sip and Grajek, 2005). Divercin AS7 is a 48-amino-acid peptide with strong antibacterial activity against *Listeria monocytogenes, Clostridium tyrobutyricum, Enterococcus faecalis* and some lactic acid bacteria strains. Divercin AS7 is resistant to heat treatment (100°C/30min and 121°C/15min), sensitive to proteolytic enzymes (pronase, proteinase K, trypsin and α-chymotrypsin), and stable over a pH rangefrom 2 to 8 (Sip and Grajek, 2005). Previous studies indicated that divercin could be used to control *Listeria monocytogenes* growth in cold-smoked fish products (salmon fillets), meat (poultry, ground pork and beef), milk, cream and dairy products. Divercin AS7 significantly reduces the level of *Listeria monocytogens* contaminations in variety of foods, therefore, it is a promising candidate for applications as a natural replacement for synthetic food- and feed-preservatives. Divercin AS7 can be introduced into feed in one of two forms: 1. as a bacteriocin preparation, more or less purified, or 2. as a starter culture producing bacteriocin *in situ* (Sip and Grajek, 2005). To our knowledge, so far there are no published results on its use in broiler chicken feed mixtures or its effect on GIT microbiota. Thus, the aim of the present trials was to evaluate divercin AS7 effects under *in vitro* conditions on GIT microflora and in an *in vivo* trial on nutrient retention, as well as the value of apparent metabolizable energy in broiler chickens.

MATERIAL AND METHODS

All procedures were approved by the Local Animal Care and Use Committee. The bacteriocin titer was expressed in activity units per ml (Arbitrary Units, AU/ml) as estimated by the critical dilution assay. The titer was defined as the reciprocal of the highest dilution showing a definite inhibition zone. The divercin AS7 preparation was produced according technology elaborated by Sip et al. (1999) and provided in the liquid form. In the *in vitro* trial, 21 male chickens were fed a provocative diet (Table 1), and at the age of 28 days were

Table 1. Composition ($g \cdot kg^{-1}$) and nutritional value of the control diet

Item	$g \cdot kg^{-1}$
Components	
triticale	210.0
wheat	467.0
soyabean meal	152.5
rapeseed meal	42.0
fish meal	30.0
rapeseed oil	43.7
monocalcium phosphate	10.8
limestone	2.9
DL-methionine 20%	13.0
L-lysine 20%	15.0
$NaHCO_3$	1.0
NaCl	2.1
mineral and vitamin premix[1]	10.0
Calculated	
ME $MJ \cdot kg^{-1}$	13.2
crude protein $g \cdot kg^{-1}$	190.0

[1] providing per kg diet, IU: vit. A 12 500, vit. D_3 4000; mg: vit. E 50, vit. K 3, vit. B_1 2.2, vit. B_2 6.5, vit. B_6 3.8, pantothenic acid 12.5, choline chloride 400, folic acid 1.5, biotin 0.2, vit. B_{12} 0.025, BHT 10, Se 0.35, Fe 60, Zn 80, Mn 80, Cu 10, I 0.75; g: Ca 2.45

slaughtered by cervical dislocation. Immediately after slaughter, the luminal contents of the crop, ileum and caeca were sampled from all of the birds and pooled by segment from 4 chickens (5 g per sample) into 7 replicates per segment. The samples were diluted in 45 ml of sterile pre-reduced salt medium (Miller and Wolin, 1974). For enumeration of bacteria, the suspension was homogenized for 2 min in CO_2-flushed plastic bags using a stomacher homogenizer (Interscience, France). To control treatment, 9 ml of pre-reduced salt medium, 8.995 ml of pre-reduced salt medium and 0.005 ml of divercin (200 $AU \cdot ml^{-1}$ the low dosage, DL), or 8.960 ml of pre-reduced salt medium and 0.04 ml of divercin (1600 $AU \cdot ml^{-1}$,

the high dosage, DH) were added to 1 ml of cell suspension. All samples were mixed and incubated for 15 min at room temperature. 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 39°C in anaerobic jars (Anaerocult A, Merck). Coliform bacteria were enumerated by spread plating on MacConkey agar (Merck 1.05465) incubated aerobically for 24 h at 39°C. The total number of anaerobic bacteria, on Columbia blood (BBL 211124) agar (CA) incubated for 48 h at 39°C in anaerobic jars (Anaerocult A, Merck).

In the *in vivo* trial, 48 one day old male ROSS 308 chickens were used. From days 1 do 18, all of the birds were kept in floor pens on straw. On day 19 the birds were randomly allocated to 4 experimental treatments, 12 birds each, and transferred to individual cages for the digestibility trial. Four diets were prepared based on a provocative diet (Table 1) in terms of stimulation of *Clostridiaceae* bacteria (Drew et al., 2004).

The non-supplemented diet (C) was the control, the SAL diet was supplemented with 70 mg/kg of salinomycin, diet DL, with 200 AU·ml^{-1} of divercin, and diet DH, with 1600 AU·ml^{-1} of divercin. Diets were offered *ad libitum* over the entire experimental period (days 1-28). In the last 5 days of the experiment, 0.3% of the wheat was replaced by titanium oxide as an internal marker for digestibility analyses. At 28 day of age, approximately 45 g of fresh excreta were collected from each bird, immediately frozen and then freeze-dried. Feed samples were analysed in duplicate for crude protein and crude fat using AOAC (2005) methods 976.05, 920.39. For chemical analyses, 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), 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) standardized with benzoic acid. Nitrogen content was analysed by the Kjel Foss Automatic 16210 (A/S N. Foss Electric, Denmark) and the protein content was calculated using a multi-plication factor of 6.25. Fat content was determined using Soxtex System HT 1043, Extraction Unit (Foss Tecator Denmark).

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

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

$$\text{AMEn (kcal/kg)} = GE_{\text{kcal/kg diet}} - [GE_{\text{kcal/kg excreta}} \cdot (TiO_2\%_{\text{diet}}/TiO_2\%_{\text{excreta}})] - 8.22 \cdot \{N\%_{\text{diet}} - [N\%_{\text{excreta}} \cdot (TiO_2\%_{\text{diet}}/TiO_2\%_{\text{excreta}})]\}$$

where: GE - gross energy, N - nitrogen, TiO_2 - titanium oxide, and 8.22 - the energy equivalent of uric acid nitrogen (i.e., 8.22 kcal/kg uric acid nitrogen).

The study was set up as a completely randomized design, and data were tested using the GLM procedure of SAS (1990). Means were separated using Duncan's multiple range test. All statements of significance are based on $P<0.05$.

RESULTS

The results of the *in vitro* and *in vivo* experiments are shown in Tables 2 and 3. In the first *in vitro* experiment, the high dose (DH) divercin supplementation (1600 AU·ml^{-1}) decreased lactic acid bacteria the in crop, ileum and caeca. In treatments DL and DH, coliforms were suppressed only in ileal contents, while the total number of anaerobes was lower ($P<0.05$) only in treatment DH in the ileum (Table 2). In the *in vivo* trial divercin AS7 supplementation tended to improve nitrogen retention and total fat digestibility ($P>0.05$). As compared with control unsupplemented treatment, the value AME_N was higher in treatment DL ($P<0.05$).

Table 2. Experiment 1. Gastrointestinal bacteria counts after divercin *in vitro* treatment, log cfu × g^{-1} wet digesta)

Treatment	Crop			Ileum			Caecum		
	MRS[1]	MCC[2]	CA[3]	MRS[1]	MCC[2]	CA[3]	MRS[1]	MCC[2]	CA[3]
C	9.43[a]	7.75[a]	9.33[a]	9.33[a]	7.94[a]	9.17[a]	9.17[a]	9.45[a]	9.50[a]
DL	9.22[a]	7.88[a]	9.10[a]	9.10[ab]	7.37[b]	9.32[a]	9.32[a]	9.08[a]	9.34[a]
DH	8.61[b]	7.52[a]	8.90[a]	8.90[b]	7.32[b]	8.41[b]	8.41[a]	8.80[b]	9.68[a]
SEM	0.020	0.107	0.110	0.021	0.264	0.270	0.594	0.186	0.137

C - control group, DL - 200 AU·ml^{-1} of divercin, DH - 1600 AU·ml^{-1} of divercin; [a,b]- means in the columns with a different letter differ significantly at $P\leq0.05$; SEM - pooled standard error of the mean, [1] - *Lactobacillus* sp., log cfu·g^{-1} digesta; [2] total anaerobic bacteria, log cfu·g^{-1} digesta; [3] - coliforms, log cfu·g^{-1} digesta

Table 3 Experiment 2. Nitrogen-corrected apparent metabolizable energy (AME_N, MJ/kg), nitrogen retention (%) and apparent total tract crude fat digestibility (%) of the diets

Item	C	SAL	DL	DH	SEM
AME_N	13.10[b]	13.33[ab]	13.68[a]	13.28[b]	0.48
N retention	58.50	60.20	61.80	62.0	3.98
Ether extract	87.10	91.10	91.40	89.10	4.29

C - control group, SAL - supplemented with 70 ppm of salinomycin, DL - 200 AU·ml^{-1} of divercin, DH - 1600 AU·ml^{-1} of divercin; [a,b] - means in the columns with a different letter differ significantly at $P\leq0.05$; SEM - pooled standard error of the means

DISCUSSION

The role of intestinal microflora in the health and disease of fast-growing broiler chickens as well as human beings is unquestioned (Guarner and Malagelada, 2003; Joerger, 2003; Ricke, 2003). Recent decades have shown rising interest of consumers in improving food chain quality and safety, specifically in terms of the elimination of foodborne pathogens like *Campylobacter, Listeria* or *Salmonella* species (Mead, 2000; Bjerrum et al., 2006). To date relatively little is known, however, on broiler chicken endogenous microflora (Bjerrum et al., 2006). Its diversity and activity is dependent on many factors, which can be divided into at least two groups: diet- and bird-associated. The first group is mostly represented by feed structure and composition as well as the presence of antibiotic growth promoters or other feed additives in a diet. The second group is correlated mainly with bird age; thus, development of the gastrointestinal tract and activity of endogenous enzymes (Knarreborg et al., 2002; Ricke, 2003; Józefiak et al., 2007). The composition of gastrointestinal microbiota also can affect nutrient absorption and fat digestibility, which influence feed utilization (Knarreborg et al., 2003; Bjerrum et al., 2006; Johansen et al., 2007). It has also been documented that antibiotic growth promoters and ionophore coccidiostats (i.e. salinomycin) lower bacterial counts in the GIT and improve feed efficiency (Knarreborg et al., 2002; Johansen et al., 2007). In contrast, little is known about the bacteriocin mode of action in the broiler chicken GIT.

Due to the presence of thick lipopolysaccharide bacterial cell walls, most of the bacteriocins produced by Gram-positive strains, including *Carnobacterium* sp., do not show activity against Gram-negative strains (Rihakova et al., 2009). In contrast, in the present *in vitro* trial, the dose of 1600 AU·ml^{-1}, which represents 0.4% of the divercin liquid preparation treatment, was characterized by the lowest lactic acid bacteria (LAB) and coliform bacteria counts (0.4-0.8 log cycle reduction) in the crop and ileal digesta. The lower dose of bacteriocin in treatment DL did not show significant antimicrobial effects. The samples were incubated with divercin for only 15 min, however, thus for a period much shorter than the *in vivo* feed passage in the broiler chicken GIT (Audisio et al., 1999). Therefore, it can be concluded that when divercin is present in the feed, its exposition in the GIT is much longer and maybe lower amounts are adequate to control the microbiota. In addition, the presented, preliminary, studies were focused on mixed cultures. Other authors showed positive effects of different bacteriocins in reducing particular pathogenic strains in the broiler chicken GIT. Stern et al. (2006) using bacteriocin secreted by *Paenibacillus polymyxa* NRRL-B-30509, demonstrated significant reduction in the colonization of the GIT by *Campylobacter jejuni*, while bacteriocin-producing *Enterococcus faecium* strain J96 showed protective effects in chickens infected with *Salmonella pullorum* (Audisio et al., 1999). In the present study there were

no differences in nutrient retention, irrespective of treatment. Salinomycin and divercin supplementation did, however, numerically increase fat digestibility and N retention. The highest AME_N of diet were determined in treatment DL. Salinomycin also improved the metabolizable energy value of the diets, but the differences were not significant when compared with the unsupplemented control group (C).

In humans, overgrowth of the bacteria that produce bile salt hydrolases in the small bowel results in steatorrhea and weight loss due to impaired digestion and absorption of lipids. In birds fed so-called provocative diets, which stimulate non-favourable microbiota populations (i.e. *Clostridiaceae*), poor feed conversions were observed due to microbial bile salt unconjugation (Knarreborg et al., 2003; Drew et al., 2004). Unconjugated bile salts are less efficient in fat digestion processes and also inhibit pancreatic lipase activity in a pH-dependent manner in the proximal small bowel of broilers (Knarreborg et al., 2002, 2003). It is possible that lower deconjugation of the bile salts was the reason for higher apparent total tract fat digestibility and partially explains the AME_N value variations. The calculated AME_N of the diets was 13.2 $MJ \cdot kg^{-1}$ (Table 1), that estimated in the balance trial was very similar (Table 3). After low-dose (0.58 $MJ \cdot kg^{-1}$) supplementation of divercin, however, improvement was observed. Salinomycin, which is known to improve nutrient retention and AME_N values (Knarreborg et al., 2002), in the present trial was also effective in those terms, but only by 0.23 $MJ \cdot kg^{-1}$. In contrast, the much higher dosage of divercin (1600 $AU \cdot kg^{-1}$ feed) did not show similar effects, even though it was more effective in reducing bacterial populations in the *in vitro* test. The achieved higher AME_N of the feed in the present trial by divercin supplementation should be considered as positive effect. In the *in vitro* trail (Table 2) it was shown that microbiota populations were dependent on the divercin dosage. Thus, it can be concluded that similar effects were present in the *in vivo* trail when the same divercin amounts were fed to birds.

CONCLUSIONS

High-dose (1600 $AU \cdot ml^{-1}$) divercin had strong bacteriostatic effects under *in vitro* conditions, however, it did not improve dietary *in vivo* apparent metabolizable energy value as much as the low dosage of 200 $AU \cdot ml^{-1}$.

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