Inhibition of *Salmonella enterica* serovar Dusseldorf by enterocin A in gnotobiotic Japanese quails

A. Laukova¹, P. Guba², R. Nemcova², M. Marekova³

¹Institute of Animal Physiology of the Slovak Academy of Sciences, Kosice, Slovak Republic
²Department of Gnotobiology and Diseases of Young, University of Veterinary Medicine, Kosice, Slovak Republic
³Department of Medical Chemistry and Biochemistry, Medical Faculty, University of P.J. Safarik, Kosice, Slovak Republic

**ABSTRACT:** The protective (ENT1) and therapeutic (ENT2) effects of enterocin A (Ent), produced by *Enterococcus faecium* EK13, against *Salmonella enterica* serovar Dusseldorf SA31 was determined in a model of gnotobiotic Japanese quails. Twenty-one 3 days old birds were divided into 3 groups of equal size; (ENT1, ENT2 and control group – CG). They were experimentally infected with SA31 (10⁷ cfu/ml) per os. For the group ENT1, Ent A (200 µl of 25 600 AU/ml) was administered 8 h before infection with SA31 strain and for the group ENT2, treatment with Ent A was administered 8 h after infection; CG was infected with SA31 and not treated with Ent A. Sampling of the feces was performed 8, 24, 48 and 168 h after infection. At the end of the experiment also the content of the caecum and ileum was analyzed. A log 1.37 reduction of SA31 colonization in feces of the group ENT1 was found after 8 h in comparison with CG. After 24 h, a significant difference in SA31 colonization was observed when comparing CG and ENT2. After 48 h, a lower colonization of SA31 was found in both groups which continued until the end of the experiment (168 h). At the same time, reduction of *Salmonella enterica* serovar Dusseldorf was detected in the content of the caecum (2.44 log) and ileum (3.16 log) in ENT2 but not in ENT 1 when compared with control group. These observations indicate stronger therapeutic effect of Ent A than prophylactic one in the digestive tract of gnotobiotic Japanese quails.

**Keywords:** *Salmonella enterica* serovar Dusseldorf; enterocin; inhibition; gnotobiotic Japanese quail

Diarrhoea caused by infectious agents is responsible for large economic losses in animal production, including chicken farms. The most frequent bacterial contaminants of domestic birds are *Salmonella* sp. or diarrheagenic *Escherichia coli*. Especially, in the case of *Salmonella* infections, these may be asymptomatic and production losses are insignificant. However, colonized birds can serve as a source of infection for humans (Tauxe, 1991) causing a disturbance of the gastrointestinal microbiota (Silva et al., 1999). Antibiotics have traditionally been the most frequently administered agents to counteract this type of infections. However, the current trend is to totally eliminate the prophylactic use of antibiotics in farming. The use of several antibiotics has already been banned (Jin et al., 2000). Therefore, in an effort to prevent or cure these disorders, alternative forms of therapy are searched. Probiotics and bacteriocin-producing strains with probiotic properties as well as bacteriocins themselves have been suggested as alternative therapies for the treatment or prevention of gastroenteritis (Elmer et al., 1996; Filho-Lima et al., 2000). *In vitro* antagonistic effects of probiotics have already been presented by several authors (Bomba et al., 1996; Jacobsen et al., 1999). Even, the effect of probiotic and bacteriocinogennic *Enterococcus faecium* EK13 strain against *Salmonella enterica* serovar Dusseldorf was already reported (Laukova et al., 2003). However, *in situ* applications of bacteriocins (especially in the GIT of animals) are supported by the Slovak Scientific Agency VEGA (Project No. 2/2043/23).
still in progress. Nisin, the most thoroughly studied bacteriocin, was used to evaluate mouth rinse in a beagle dog model, and was shown to prevent gingival inflammation (Howel et al., 1993; Delves-Broughton et al., 1996). Bacteriocins are defined as proteinaceous compounds that kill or inhibit more or less related bacterial genera (Klaenhammer, 1993; Nes et al., 1996). The most frequent bacteriocin-producing strains were detected among lactic acid bacteria, including enterococci (Nes et al., 1996).

Enterocins, bacteriocins produced by enterococci have been studied in more detail during the last decade (Laukova et al., 1993; Aymerich et al., 1996; Casaus et al., 1997; Floriano et al., 1998; Marekova et al., 2000). Enterococcus faecium EK13 is a strain which produces enterocin A (Ent A) (Marekova et al., 2003). The aim of the current study was to determine in a model of gnotobiotic Japanese quails the preventive and therapeutic effects of Ent A against an experimental infection with Salmonella enterica serovar Dusseldorf. Gnotobiotic animals provide a convenient in vivo model to assess the efficacy of Ent A as well as for comparing the results with those from conventional animals. In spite of the fact, that Salmonella enterica serovar Dusseldorf is not common contaminant in poultry, the strain SA31 was used because it was inhibited by enterocin A under in vitro conditions (Vasilkova et al., 2002). The inhibitory effect of Lactobacillus sp. AD1 strain against SA31 strain in Japanese quails was also noticed by Strompfova et al. (personal communication). Japanese quails represent well managing animal model; that’s why they were chosen for our experiment.

MATERIAL AND METHODS

Gnotobiotic Japanese quails were obtained by disinfection of eggs with 2% formaldehyde in air. The eggs were subsequently transferred to sterile gnotobiotic cabinets. The group of twenty-one 3-days old Japanese quails was divided into three working groups-seven birds in each. Salmonella enterica serovar Dusseldorf SA31 (a generous gift from Dr. Zuzana Vasilkova, Parasitological Institute of the Slovak Academy of Sciences, Kosice, Slovakia) was grown in trypticase soy broth (Becton & Dickinson, Cockeysville, USA) at 37°C (pre-cultured twice). Crude enterocin A (Ent A, 25 600 AU/ml) produced by E. faecium EK13 was prepared as described previously by Marekova et al. (2003). Briefly, EK13 strain was grown for 18 h at 37°C in MRS broth. Cell-free supernatant fluid (pH adjusted to 5.5) was collected by centrifugation (30 min at 10 000 g), treated by EDTA, heated for 30 min at 80°C, cooled and concentrated at rotary evaporator. Activity of crude enterocin A was tested according to De Vuyst et al. (1996).

The birds were fed with sterilized feeding mixture BR1 (Tatrat s. r., Huncovce, Slovakia) and had access to water ad libitum. All three groups of birds, control group (CG), experimental group 1 (ENT1) and experimental group 2 (ENT2) were infected with 200 µl of S. enterica serovar Dusseldorf SA31 culture (10⁷ cfu/ml) per os. To determine the protective effect of Ent A, 200 µl of the preparation was applied to group ENT1 eight hours before challenge with SA31 strain. To test therapeutic effect of Ent A, group ENT2 received the bacteriocin preparation 8 h after SA31 infection. Feces were sampled at 8, 24, 48 and 168 h after infection. After 168 h, the birds were sacrificed and the level of SA31 was determined in the caecal and ileal contents by plating serial dilutions on brilliant green agar (BGA, Becton & Dickinson). Plates were incubated at 37°C for 48 hours.

Statistical analyses were performed with a two sided unpaired Student’s t-test with the level of significance set at P < 0.05. Differences in Figures 1 and 2 are indicated by the same letters and expressed significance of P < 0.01 and P < 0.001.

RESULTS AND DISCUSSION

Comparing the total counts of SA31 strain in feces of gnotobiotic Japanese quails after 8 h between the group ENT1 (Figure 1) and CG, a difference of log 1.37 was detected (P < 0.001). After 24 h, a significant difference (P < 0.001) was observed when comparing CG and ENT2 (Figure 1). After 48 h, a lower level of colonization by SA31 in the both experimental groups was detected (P < 0.01 for ENT1 and P < 0.001 for ENT2) which continued until the end of the experiment (168 h, Figure 1). The level of colonization by SA31 in the caecal and ileal contents of quails in the ENT2 group after 168 h was reduced by log 2.44 (P < 0.001) in the caecum and 3.16 log in the ileum (P < 0.001, Figure 2) compared to CG. Colonization of quails by SA31 was not different in the caecum nor in ileum after 168 h in the ENT1 group compared with the CG.

Ent A reduced the colonization of Salmonella enterica serovar Dusseldorf SA31. The most effective
inhibition was found with the therapeutic application of Ent A in comparison with its prophylactic effect. Up to now, enterocins have experimentally been applied to different matrices such as food and dung (Nunez et al., 1997; Laukova et al., 2002). The observations presented here suggests that they could find application in the digestive tract as well, to reduce the levels of undesirable microbes such as salmonellae (Audisio et al., 1999, 2000). Reduction of Salmonella enterica serovar Dusseldorf in pig slurry (1 order of magnitude) was also reported by Vasilková et al. (2002). The results of the current study indicate that further investigations for bacteriocin application in the ecosystem mentioned are warranted. It is not clear why the therapeutic effect is much stronger than the prophylactic one. However, it is possible that the effect of enterocin is gradually influenced by proteolytic enzymes in the GIT. Therefore, if applied 8 hours prior the infection, the inhibitory effect of enterocin can be already diminished. Similar results to the application of the bacteriocin producer were obtained when the producer E. faecium EK13 strain was used for precolonization or superinfection of gnotobiotic Japanese quails except for the fact that no inhibition was found in the caecum and ileum at the end of the experiment (Laukova et al., 2003). Crude bacteriocin is therefore of slightly stronger effect than the application of producer strain itself. This might be caused by the low ability of the strain to adhere, transiently colonize the digestive tract and produce the enterocin in vivo. Similar findings were presented by Maia et al. (2001) who reported a protective effect of E. faecium (component of Vitacanis, a probiotic preparation to prevent the intestinal disorders in dogs and cats) against an experimental challenge with Salmonella enterica serovar Typhimurium, despite the fact that protective effect was not due to the reduction of intestinal populations of the pathogenic bacteria. Probiotic effect of live E. faecium strains can be thus caused by mechanisms independent of bacteriocin production, perhaps as a consequence of rapid influx of neutrophils as a response to the first contact of intestinal mucosa with bacteria (Salminen et al., 1998; Waar et al., 2002). Although EK13 strain has also probiotic properties, its bacteriocin mediated effect is here dominating.
That is, it is a basic difference among the results reported here and those already reported by Laukova et al. (2003) in gnotobiotic Japanese quails; when directly EK13 strain (enterocin A-producing) was effectively tested in its inhibition against Salmonella enterica serovar Dusseldorf (former mentioned). And, there testing prophylactic application, significant reductions of SA31 strain were found in feces at 24 and 48 hours as well as in caecum; however not in ileum. From the results obtained could be also concluded, that although bacteriocin production is one among selection criteria for probiotic strains, it doesn’t mean that the strain without bacteriocin production cannot be effective probiotic. Because of its many other probiotic properties. On the other hand, bacteriocin-producing strain itself can also successfully influence microbial society in the ecosystem by its bacteriocin. However, combinations of the properties, e.g. bacteriocinogenic and probiotic characters of the strain indicates its higher final effectivity.

Acknowledgements

We wish to thank Ms. Janka Karafova for her skillful technical assistance. We are also grateful to Dr. Vladimir Lasanda from the State Veterinary Institute in Kosice (Slovakia) for typing of strain SA31. Our greatest thanks belongs to Dr. Arthur Ouwehand from University of Turku, Department of Biochemistry and Food Chemistry (Finland) for his kindness to check and to improve our manuscript.

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Received: 03–05–20
Accepted after corrections: 03–11–18

Corresponding Author

MVDr. Andrea Laukova, Institute of Animal Physiology, Slovak Academy of Sciences, Soltesovej 4–6, 04001 Kosice, Slovak Republic
Tel. +421 55 633 02 83, fax +421 55 728 78 42, e/mail: laukova@saske.sk