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

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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 (10^7 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 countract 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

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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 convient *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 group1 (ENT1) and experimental group 2 (ENT2) were infected with 200 µl of *S. enterica* serovar *Dusseldorf* SA31 culture (10^7 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 SA 31 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 illeal 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

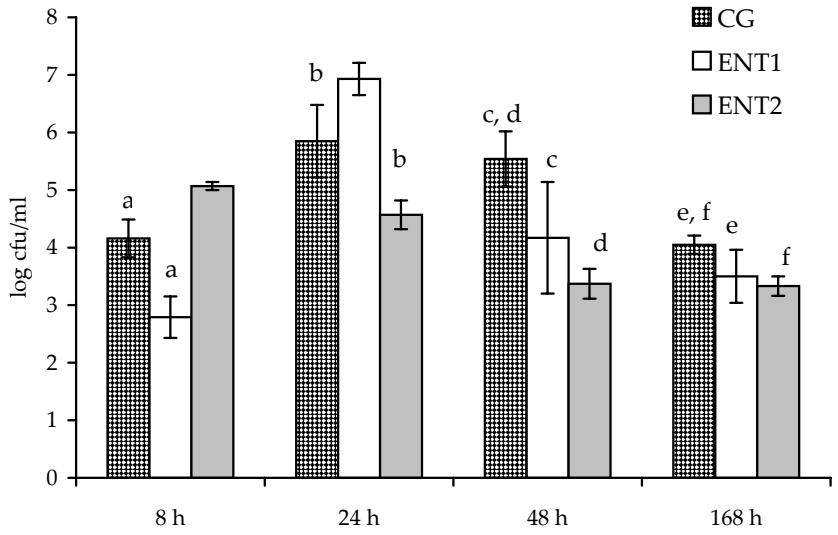

Figure 1. Counts of *Salmonella enterica* serovar *Dusseldorf* in feces of gnotobiotic Japanese quails treated with enterocin A 8 h before infection (ENT1), 8 h after infection (ENT2) or untreated (CG)

a, b, d, f = $P < 0.001$; c, e = $P < 0.01$

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 Vasilkova *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 indepent 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.

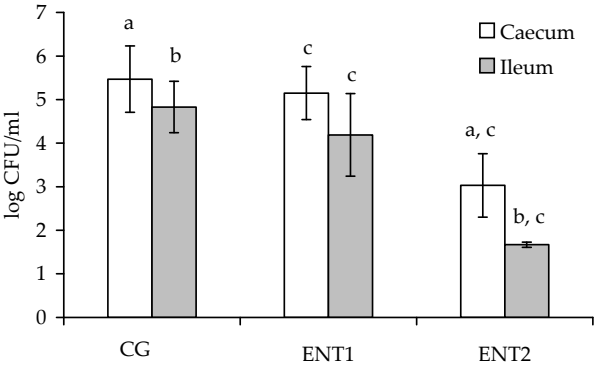

Figure 2. Counts of *Salmonella enterica* serovar *Dusseldorf* in caecum and ileum of gnotobiotic Japanese quails 168 h after infection and treatment with enterocin A. Birds were treated with enterocin A either 8 h before infection (ENT1) or 8 h after infection (ENT2), or were left untreated (CG)

a, b, c = $P < 0.001$

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 effectivelly 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. bacteriocinogennic and probiotic characters of the strain indicate its higher final effectivity.

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