

## Effect of Bacteriocin-Like Substance Produced by *Enterococcus faecium* EF55 on the Composition of Avian Gastrointestinal Microflora

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### Abstract

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The influence of daily orally administered crude extract of bacteriocin-like substance (at a concentration of 3200 AU/ml) produced by *Enterococcus faecium* EF55 on the total counts of lactobacilli, staphylococci, enterococci and *E. coli* in the faeces and caecum of 3-day-old Japanese quails was investigated. *Enterococcus faecium* EF55 was isolated from the crop content of a chicken (*Gallus domesticus*). The inhibitory activity of bacteriocin-like substance (BLIS) produced by the strain EF55 was assayed by the agar spot test using Gram-positive and Gram-negative indicator bacteria. A wide range of Gram-positive genera such as *Enterococcus*, *Staphylococcus*, *Micrococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus* and *Aerococcus* was susceptible to BLIS, but none of the Gram-negative bacteria. The antimicrobial substance produced by the strain EF55 was thermo-resistant (30 min at 100 °C), stable at pH 4.0 to 9.0 at -20 °C, 4 °C and 22 °C for 10 d tested, and inactivated by proteolytic enzymes indicating its proteinaceous nature. After the first administration of bacteriocin crude extract (BCE) of EF55 strain to Japanese quails, a reduction amounting to 0.83–1.3 log cycles of *E. coli*, enterococci, staphylococci and lactobacilli in faeces was observed within 24 h. This inhibitory effect was most visible after first extract addition, later this difference was diminished. By agar spot test, BLIS produced by the strain EF55 of *Ent. faecium* was active against *Staphylococcus* spp., *Lactobacillus* spp. and *Enterococcus* spp., isolates obtained from the experimental birds. However, no inhibition against *E. coli* was detected, despite of their decreased counts under *in vivo* conditions.

*Bacteriocin, Japanese quails, gastrointestinal microflora, effect*

The ability of bacteria to produce bacteriocins is well known (Klaenhammer et al. 1993). Bacteriocins are proteinaceous compounds with inhibitory activity against more or less related bacterial genera (Nes et al. 1996). Bacteriocin-producing bacteria have been isolated from a variety of habitats (Dykes 1995) and bacteriocins have been characterized from many different bacterial genera, especially from lactic acid bacteria (Jack et al. 1995). Nisin, produced by certain strains of *Lactococcus lactis* subsp. *lactis*, is the best characterized bacteriocin-lantibiotic widely used for decades as a food preservative (Delves-Broughton et al. 1996). More recently, also biomedical applications have been proposed which include its potential to treat mastitis infections in cows (Sears et al. 1995), gingivitis and gastrointestinal infections such as peptic ulcer disease caused by *Helicobacter pylori* (Blackburn and Projan 1994). Nisin has been proved to modulate the immune system of mice when included in the diet (De Pablo et al. 1999) or of turbot after its intraperitoneal injection (Villamil et al. 2002). Strains of enterococci, including *Enterococcus faecium* and *Ent. faecalis*, have also the ability to produce bacteriocins. Numerous studies concerning enterococci (bacteriocins produced by enterococci) have been published during recent ten years. Up to now the following enterococci have been characterized as to their homogeneity: enterocin A (Aymerich et al. 1996), enterocin

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B (Casaus et al. 1997), enterocin P (Cintas et al. 1997), enterocin I (Floriano et al. 1998), enterocin M, a new variant of enterocin P (Mareková et al. 2002), and enterocin CCM 4231 (Lauková et al. 1997). Whereas the effectiveness of enterocins against food spoilage and pathogenic bacteria in various food systems is well demonstrated (Aymerech et al. 2000), little information is available on their possible antagonistic activity in the intestine *in vivo*. Therefore, the objective of this study was to determine the effect of orally administered bacteriocin-like substance (crude extract), produced by the isolate EF55 of *Enterococcus faecium*, on the composition of intestinal microflora of conventional Japanese quails. Additionally, antimicrobial activity of this strain against a number of Gram-positive and Gram-negative bacteria was tested.

### Materials and Methods

#### Experimental animals

A group of fourteen 3-day-old Japanese quails was divided into two groups of seven birds each. The experiment lasted for seven days. All birds were fed the commercial diet BR 1/FAT (Tatrafat s.r. Huncovce, Slovakia) and had access to water *ad libitum*. The experimental group (n = 7) was orally administered the crude bacteriocin extract of *Ent. faecium* EF55 at a concentration of 3200 AU/ml (one oral dose 0.10-0.25 ml) every 24 hours. The control group (n=7) was given placebo – Brain heart infusion (BHI) broth (Becton & Dickinson, Cockeysville, USA). Sampling of faeces was done from each quail after 1, 3 and 7 d to monitor the effect of the bacteriocin crude extract (BCE) on the microflora of their gastrointestinal tract. At the end of the experiment all animals were sacrificed and their caecum was separated. Its content was mixed using Stomacher (801, England). The birds were weighed both at the beginning and at the end of the experiment.

#### Bacterial counts

The samples of faeces and caecum content were serially diluted in saline buffer (0.85%) according to the standard microbiological method and plated on the following media: Mac Conkey agar (Imuna, Šarišské Michaľany, Slovakia) for enumeration of *E. coli*, Mannitol salt agar (Becton & Dickinson) for staphylococci, M-*Enterococcus* agar (Becton & Dickinson) for enterococci and Rogosa agar (prepared according to Oxoid manufacturer, pH 6.2) for lactobacilli. Enterococci, staphylococci and *E. coli* were cultivated at 37 °C for 24 and 48 h, respectively. Lactobacilli were cultivated in a 3% CO<sub>2</sub> atmosphere at 37 °C for 48 h. Numbers of colony forming units (cfu) were expressed as log 10 cfu per gram. The results are given as arithmetical means ± S.E.M.

#### Preparation of crude extract

The bacteriocinogenic strain EF55 of *Ent. faecium* was grown in 300 ml of BHI broth (Becton & Dickinson) at 37 °C for 18 h (O.D.<sub>600</sub> 1.1). Culture supernatant was collected by centrifugation (10 000 g for 30 min), neutralized and concentrated in a rotary evaporator. The activity of crude extract was determined by the agar spot test (De Vuyst et al. 1996) on BHI agar plates (1.5 and 0.7%; Becton & Dickinson) and expressed in arbitrary units per ml of culture medium (AU/ml). Arbitrary unit is defined as the reciprocal of the highest dilution giving growth inhibition of the indicator organism. As the indicator was used EA5 strain of *Enterococcus avium*.

#### Antimicrobial activity assays

The inhibitory activity of the cell-free supernatant of *Ent. faecium* EF55 was assayed by the agar spot test (De Vuyst et al. 1996) on BHI agar plates (1.5 and 0.7%; Becton & Dickinson) against the target of Gram-positive and Gram-negative bacteria. A complete list of indicator bacteria used in this study is given in Table 1. The bacteriocin activity of crude extract against strains isolated from the faeces of the experimental quails by using selective growth media mentioned above was also tested by the agar spot test.

#### Effect of enzymes, pH and heat on bacteriocin activity

Sensitivity to proteolytic enzymes was tested individually at a concentration of 1 mg/ml by adding of the following enzymes to cell-free supernatant of *Ent. faecium* EF55: protease (Sigma), trypsin (Sigma),  $\alpha$ -chymotrypsin (Sigma). Reaction mixtures were incubated at 37 °C for 1 h.

The thermostability of the antibacterial activity was determined by heating of cell-free neutralized supernatant fluids at 60 °C, 80 °C and 100 °C for 30 min. To determine the effect of pH on bacteriocin activity, cell-free supernatant fluid was adjusted to pH levels ranging from 4.0 to 9.0 (intervals of 1.0) with 1N HCl or 5N NaOH. The pH-adjusted supernatants were kept at -20 °C, 4 °C and room temperature (22 °C) for 10 d. Bacteriocin activity of all tests was determined using agar spot test method and *Ent. avium* EA5 as indicator.

#### Statistical analysis

Statistical evaluation of the results was performed by the Students' *t*-test with the level of significance set at  $P < 0.05$ .

## Results and Discussion

*Enterococcus faecium* EF55 (own isolate) was isolated from the crop content of a chicken. It produces a bacteriocin-like substance with the inhibitory activity against Gram-positive bacteria including enterococci, staphylococci, lactococci, streptococci, lactobacilli, micrococci, but no inhibition against Gram-negative bacteria tested *in vitro* was detected (Table 1). This observation correlated with numbers of enterocins studied (Giraffa 1995). The antimicrobial substance was thermo-stable (30 min at 60 °C, 80 °C and 100 °C) and it was stable over a pH

Table 1  
Inhibitory spectrum of bacteriocin-like substance produced by strain EF55 against indicator strains

Indicator organism	Source	Activity	
<b>Gram-positive bacteria</b>			
<b>Enterococcus spp.</b>	<i>E. avium</i> EA5	faeces of piglet	+
	<i>E. casseliflavus</i> EC7	faeces of J. quails	+
	<i>E. casseliflavus</i> EC24	rumen content of deer	+
	<i>E. durans</i> 5A	faeces of piglet	+
	<i>E. faecalis</i> EE P4	faeces of J. quails	+
	<i>E. faecalis</i> EE61	faeces of piglet	+
	<i>E. faecalis</i> V24	dung water	+
	<i>E. faecium</i> AL41	dung water	+
	<i>E. faecium</i> CCM 4231	rumen content of calf	+
	<i>E. faecium</i> EF 43	faeces of pig	+
	<i>E. faecium</i> M74	faeces of infant	+
<b>Staphylococcus spp.</b>	<i>S. aureus</i> SA5	fish salad	–
	<i>S. aureus</i> SA105	milk-mastitis	+
	<i>S. aureus</i> SA2	fish salad	+
	<i>S. chromogenes</i> SR3	rumen content of deer	–
	<i>S. lentus</i> SL163	faeces of deer	+
	<i>S. xylosus</i> SX310	rumen content of calf	+
	<i>S. warneri</i> SW28	cattle dung water	+
<b>Micrococcus spp.</b>	<i>M. luteus</i> ML10	milk-mastitis	–
	<i>Micrococcus</i> sp. 4982	fish salad	+
<b>Lactobacillus spp.</b>	<i>Lb. acidophilus</i> LA99	vegetable salad	+
	<i>Lb. johnsonii</i> LJ4982	vegetable salad	–
<b>Lactococcus spp.</b>	<i>Lc. lactis</i> 96RS	from collection <sup>a</sup>	+
	<i>Lc. lactis</i> 1391	from collection <sup>a</sup>	+
<b>Streptococcus spp.</b>	<i>Str. bovis</i> A024/85	rumen content of calf <sup>b</sup>	+
	<i>Str. bovis</i> SB 357	from collection <sup>a</sup>	+
	<i>Str. crista</i> SC 1898	fish salad	+
<b>Aerococcus spp.</b>	<i>Aerococcus viridans</i> ESc51	from collection <sup>a</sup>	+
<b>Gram-negative bacteria</b>	<i>Salmonella dusseldorf</i> SA31	faeces of pig <sup>c</sup>	–
	<i>E. coli</i> Ax105	human clinical isolate <sup>d</sup>	–
	<i>Enterobacter georgiviae</i> EG3	dung water	–
	<i>Klebsiella</i> sp. On 160	human clinical isolate <sup>d</sup>	–
	<i>Providencia alcalifaciens</i> P4	dung water	–

+ inhibition of indicator strain

– negative reaction

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range of 4.0–9.0 at –20 °C, 4 °C and room temperature (22 °C) for 10 d tested. The treatment with proteolytic enzymes (trypsin,  $\alpha$ -chymotrypsin, protease) resulted in the loss of antibacterial activity, which confirmed the proteinaceous character of this substance. On the basis of these results, it is thermo-stable proteinaceous substance with a stability over a wide range of pH values such as most of enterococci (Giraffa 1995). Activity of bacteriocins over a wide pH range is an important asset, allowing their use under acidic conditions (e.g. small intestine, fermented milks) as well as in more neutral environments (large intestine, low-acid foods).

The crude bacteriocin extract of strain EF55 (at a concentration of 3200 AU/ml) was used in the experiment *in vivo*. It was daily orally administered to each quail during seven consecutive days. The total counts of selected bacterial groups in the faeces of birds with developing intestinal microflora are summarized in Table 2. After 24 h of crude extract addition, the total counts of all

Table 2  
*In vivo* effect of bacteriocin crude extract produced by the strain EF55 of *Ent. faecium* on bacteria in the gastrointestinal tracts of Japanese quails

Day	Log 10 cfu/g of faeces							
	<i>E. coli</i>		<i>Enterococcus spp.</i>		<i>Lactobacillus spp.</i>		<i>Staphylococcus spp.</i>	
	Control group	Exper. group	Control group	Exper. group	Control group	Exper. group	Control group	Exper. group
0	6.82 (0.41)		6.67 (0.37)		7.84 (0.46)		3.52 (0.34)	
1	8.12 (0.30) <sup>a</sup>	7.09 (0.31)*	7.79 (0.41)	6.63 (0.33)*	8.36 (0.29):	7.06 (0.22)**	3.83 (0.30)	3.00 (0.41)
3	8.62 (0.27)	7.72 (0.39)	8.00 (0.15)	7.73 (0.37)	9.12 (0.32)	7.81 (0.35)*	4.42 (0.35)	3.41 (0.30)*
7	8.54 (0.30)	7.73 (0.31)	7.96 (0.27)	7.61 (0.39)	9.10 (0.38)	7.80 (0.49)	3.97 (0.48)	3.75 (0.38)
7 <sup>b</sup>	8.71 (0.27)	8.53 (0.35)	8.12 (0.37)	7.86 (0.33)	9.30 (0.46)	8.10 (0.45)	3.71 (0.47)	3.10 (0.26)

<sup>a</sup> the values in parentheses are standard errors means

<sup>b</sup> Total bacterial count detected in caecum content

\*  $P < 0.05$

\*\*  $P < 0.01$

bacterial groups under study (enterococci, staphylococci, lactobacilli and *E. coli*) were lower compared with control group. The greatest cell count reduction (significant difference) was detected after the first administration of BCE except for the greatest reduction of total counts of staphylococci (difference of 1.01 log;  $P < 0.05$ ) detected 72 h after first BCE administration. Lactobacilli were significantly ( $P < 0.01$ ) reduced 24 h after first BCE application (difference of 1.30 log), and this reduction was relatively stable during the experiment. The total count of enterococci was significantly ( $P < 0.05$ ) lower only after the first BCE application (difference of 1.16 log). The difference in the counts of *E. coli* between experimental and control groups was 1.03 log ( $P < 0.05$ ) also on d 1, later it was diminished. Concerning the total counts of cells, staphylococci were the most inhibited bacterial group (reduction of 22.9%), followed by lactobacilli (15.6%), enterococci (14.9%) and *E. coli* (12.7%). In the contents of caecum, no significant difference in the total counts of bacterial genera was detected. No differences in body mass between the control and experimental groups were noted.

By the testing of BCE of EF55 *in vitro* against the isolates from the faeces of quails obtained before experimental treatment (Table 3), a similar, most pronounced inhibitory effect was noted against *Staphylococcus spp.*, followed *Lactobacillus spp.* and *Enterococcus spp.* Surprisingly, no inhibition against the isolates of *E. coli* was detected, despite of their reduction under *in vivo* conditions. This could be due to secondary interactions between intestinal microflora groups. Moreover, activity of enterococci towards Gram-negative bacteria like *E. coli* (Tomita et al. 1997) and *Vibrio cholerae* has been shown (Simonetta et al. 1997).

Many bacterial species inhabiting the animal gut produce compounds that are inhibitory, *in vitro*, to the growth of other bacteria (Du Toit et al. 2000). Many investigators have considered

Table 3  
*In vitro* inhibition of bacteriocin crude extract of EF55 strain against isolates from the faeces of experimental J. quails

Species	Number of strains	
	tested	inhibited, %
<i>Staphylococcus</i> spp.	15	73.3
<i>Lactobacillus</i> spp.	15	33.3
<i>Enterococcus</i> spp.	15	26.7
<i>Escherichia coli</i>	15	0.0

the possibility that these compounds influence the survival of a bacterium in an ecological niche in the gastrointestinal tract of animals. In our experiment, the bacteriocin-like substance produced by the strain EF55 of *Ent. faecium* was able to reduce the populations of present intestinal microflora in experimental birds, although this effect was especially remarkable after its first oral administration. Similarly, this type of inhibition kinetics has been observed before with *Listeria* as indicator and nisin, pediocin AcH and enterococin EFS2 as antimicrobial substances (Song and Richard 1997), and may indicate that sensitive bacteria become resistant to the bacteriocin. It is generally admitted that each bacteriocin-sensitive bacterial population includes potentially tolerant and/or resistant cells with structural modifications or at least with a high predisposition to such modifications, which would allow them to spontaneously emerge in case of exposure to the bacteriocin (Hanlin et al. 1993). In particular, modifications in the cytoplasmic membrane composition are often investigated to explain bacteriocin resistance, considering the key role of the membrane in the activity of bacteriocins (Mazzotta and Montville 1997). Because the occurrence of bacteriocin tolerance and/or resistance is the main limiting factor for bacteriocin effectiveness, the use of a combination of several bacteriocins could prevent or delay the apparition of bacteriocin-resistant mutants (Morency et al. 2001).

In conclusion, bacteriocins produced by intestinal *Enterococcus* isolates may help to control the autochthonous microflora and may be advantageous to the producing strain for its establishment and competition in the gastrointestinal tract. Further studies on bacteriocins behaviour in the intestinal environment are required to determine their suitability for potential biomedical applications where they may provide valuable alternatives to antibiotics for the treatment of animal infections without any side effects and leaving residues in the animal products behind.

#### Účinek bakteriocin-like substancie produkovanej kmeňom *Enterococcus faecium* EF55 na zloženie gastrointestinálnej mikrofóry

V práci bol sledovaný účinok orálne aplikovaného hrubého extraktu bakteriocin-like substancie (konc. 3200 AU/ml), produkovanej kmeňom *Enterococcus faecium* EF55, na celkové počty laktobacilov, stafylokokov, enterokokov a *E. coli* v truse a céku trojdňových japonských prepelíc. *Enterococcus faecium* EF55 bol izolovaný z obsahu hrvoľa kurčáťa. Pomocou agar spot testu bola sledovaná inhibičná aktivita bakteriocin-like substancie (BLIS) proti niektorým Gram-pozitívnym a Gram-negatívnym baktériám. Na túto substanciu bolo citlivé široké spektrum Gram-pozitívnych baktérií, ako sú baktérie z rodu *Enterococcus*, *Staphylococcus*, *Micrococcus*, *Lactobacillus*, *Lactococcus*, *Streptococcus* a *Aerococcus*, ale žiadna z Gram-negatívnych baktérií. Bakteriocin-like substancia je relatívne termo-rezistentná (30 min pri 100 °C), stabilná pri pH 4,0–9,0 a súčasne teplote -20 °C, 4 °C a 22 °C počas 10 dní testovania a inaktivovateľná proteolytickými enzýmami, čo poukazuje na jej proteínový charakter. U prepelíc bola 24 h po prvej aplikácii tejto substancie pozorovaná redukcia v rozsahu 0,83–1,3 log u všetkých sledovaných skupín baktérií (enterokoky, laktobacily, stafylokoky, *E. coli*). Inhibičný účinok bol najzreteľnejší po prvej aplikácii, neskôr postupne slabol. Podobne bola zaznamenaná inhibičná aktivita *in vitro* (agar spot test) proti rodom *Staphylococcus*, *Lactobacillus* a *Enterococcus* – izolátom z pokusnej skupiny zvierat. Inhibícia proti *E. coli* nebola detegovaná, napriek ich poklesu za *in vivo* podmienok.

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