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Selection of enterococci for potential canine probiotic additives

Viola Strompfová[a,*], Andrea Lauková[a], Arthur C. Ouwehand[b]

[a] *Institute of Animal Physiology, Slovak Academy of Sciences, Šoltésovej 4-6, 04001 Košice, Slovak Republic*
[b] *Department of Biochemistry and Food Chemistry, University of Turku, Vatselanjatu 2, 20014 Turku, Finland*

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Abstract

Enterococci are important inhabitants of animal intestine and are widely used in probiotic products. A potentially successful probiotic strain is expected to have several desirable properties in order to be able to exert its beneficial effects. Forty enterococcal isolates from dog faeces were tested for characters believed to be important for probiotic strains; bacteriocin production, resistance or tolerance to antibiotics, low pH, bile tolerance and adhesive activity. The total count of enterococci was found to be 3.3–7.3 $\log_{10}$ CFU/g of faeces. Most identified strains were *Enterococcus faecium*. All strains were sensitive to vancomycin, ampicillin, penicillin and chloramphenicol. Thirty-three percentage of strains were resistant to erythromycin and 28% to tetracycline. Among 40 isolates, 75% showed a broad inhibitory spectrum only against Gram-positive indicator bacteria. Seven strains with broad bacteriocin activity were selected for further assays. In the presence of 1% bile, the survival rate of selected strains ranged between 72 and 98%. Survival of strains at pH 3.0 was found in the range between 76 and 87% after 3 h. The adhesion of the tested strains to intestinal mucus ranged from 4 to 11% for canine mucus and from 5 to 8% for human mucus. *E. faecalis* EE4 and *E. faecium* EF01 showed the best probiotic properties. It indicates that they could be used as new candidate probiotic strains after in vivo testing.

Keywords: *Enterococcus* sp.; Probiotic; Identification; Adhesion; Antibiotic; Acid and bile tolerance; Antimicrobial substances

1. Introduction

Enterococci have a widespread presence in the environment and also belong to the enteric commensal microbiota (Devriese et al., 1992). Many produce the antimicrobial substances (enterocins) and have an inhibitory effect on spoilage organisms (Sabia et al., 2002; Cintas et al., 1997). Their bacteriocinogenic effect has been experimentally utilized in different ecosystems (Nunez et al., 1997; Lauková and Czikková, 1998). Moreover, enterococci are used as probiotic organisms because of their good growth, adhesive ability, lactic acid production and the stability of their enterocins (Maia et al., 2001).

Probiotics are defined as direct feed microbials or microbial cell preparations with a beneficial effect on the health and well-being of the host (Nemcová, 1997). The majority of the commercially available probiotic preparations for animals are composed of species from the genera *Bacillus*, *Enterococcus*, *Lactobacillus*, *Streptococcus* as well as from yeasts such as *Saccharomyces* spp. Among enterococci, *Entero-*

* Corresponding author. Tel.: +421-55-6330283; fax: +421-55-7287842.
E-mail address: strompfv@saske.sk (V. Strompfová).

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coccus faecium is the most frequently used species in commercial probiotics, but more attention is currently also directed towards *Enterococcus faecalis* as a probiotic (Ozawa et al., 1983). *E. faecium* strains used as probiotics efficiently protect animals from diseases caused by *E. coli*, salmonellae or clostridia (Maia et al., 2001). Before using an organism as a probiotic several properties may prove useful in evaluating the potential of the bacteria; strain origin, acid and bile tolerance, adhesion to the intestinal mucus, production of antimicrobial substances and antibiotic resistance or sensitivity (Salminen et al., 1998). The importance of selecting probiotic strains from the homologous species is uncertain as the adhesive activity of enterococci to mucus from different animal species differs (Rinkinen et al., 2000; Lauková et al., in press).

A cause of intestinal disorders in dogs is small intestinal bacterial overgrowth (SIBO). This involves increased numbers of a mixed flora, most commonly with the members of the family *Enterobacteriacae* as well as with *Bacteroides* spp., *Clostridium* spp., enterococci and staphylococci (Rutgers et al., 1995). Maia et al. (2001) reported the best preventive effectivity of *E. faecium* from commercial probiotic preparation Vitacanis (composed from *L. acidophilus*, *E. faecium* and *S. cerevisiae*) against *S. enterica* ser. Typhimurium in gastrointestinal tract of gnotobiotic mice. However, up to now, few studies have been performed in dogs by use of already known probiotic strains (Zentek et al., 1998; Benyacoub et al., 2003). Therefore, the aim of this study was to isolate and to select the most promising *Enterococcus* strains from canine faeces for their further detailed characterization as well as for in vivo experiments in dogs.

2. Materials and methods

2.1. Bacterial isolation

Faeces of 10 healthy dogs of eight breeds, both sexes with median age 2 years (in the range from 3 months to 6 years) were serially diluted in saline solution (pH 7.0), plated on M—*Enterococcus* agar (Becton and Dickinson, Cockeysville, MD, USA) and incubated at 37 °C for 48 h. Forty colonies were randomly picked and maintained on Brian Heart agar (1.5%; Becton and Dickinson) for further identification and testing.

2.2. Species identification

For DNA preparation all strains were cultivated on Slanetz–Bartley agar (Oxoid, Basingstoke, Hampshire, UK) at 37 °C in a 5% CO_2 atmosphere for 24 h and checked for purity. DNA was prepared as described by Baele et al. (2000). To identify enterococci tDNA-intergenic PCR (tRNA-intergenic length polymorphism analysis) according to Baele et al. (2000) was used followed by capillary electrophoresis (Welsh and McClelland, 1991). tRNA-gene consensus primers T5A (5′AGTCCGGTGCTCTAACCAACTGAG and T3B (5′AGGTCGCGGGTTGGAATCC) were used for PCR in order to amplify the intergenic regions between tRNA genes, as described by Welsh and McClelland (1991). Electropherograms were interpreted visually and with a software program developed and described by Baele et al. (2000). The software compares samples which are derived from the ABI 310 Genescan Analysis programme as PCR fragments length tables.

2.3. Sensitivity or resistance of isolates to antibiotics, low pH and bile tolerance

Antibiotic sensitivity/resistance of the isolates was tested by the agar disc diffusion method and following discs (Becton and Dickinson) were used: ampicillin (10 μg), chloramphenicol (30 μg), erythromycin (15 μg), gentamicin (10 μg), kanamycin (30 μg), penicillin G (10 IU), tetracycline (30 μg), and vancomycin (10 μg). *E. faecalis* ATCC 29212 was used as the control strain.

Tolerance of isolates against bile was tested according to Gilliland and Walker (1990). Briefly, overnight cultures were inoculated (2%) into MRS broth (Becton and Dickinson) without and with oxgall (1%, Becton and Dickinson) added and incubated at 37 °C. The number of viable enterococci was determined at 0 h and after 24 h of incubation, on MRS agar plates. To test survival of the isolates at low pH values, the cells of overnight cultures of selected strains (MRS broth, Becton and Dickinson) were harvested by centrifugation (at 2000 × *g* for 15 min), resuspended in 0.05 M phosphate buffer—pH 3.0 adjusted with 1N HCl, and held at 37 °C for 1–3 h. The colony forming units were determined on MRS agar.

2.4. Mucus adhesion assay

Human intestinal mucus was isolated from the healthy part of resected colonic tissue as described earlier (Ouwehand et al., 1999). In short, resected tissue was gently washed in PBS containing 0.01% gelatin and mucus was collected by gently scraping the mucosa with a rubber spatula. The mucus was centrifuged at 13,000 × *g* for 10 min to remove cell debris and bacteria and stored at −80 °C until use. Canine mucus was prepared from canine jejunal chyme essentially as described (Kirjavainen et al., 1998; Ouwehand et al., 1999). Briefly, jejunal chyme was centrifuged at 12,000 × *g* to remove particulate matter. Mucus was precipitated from the clear supernatants by dual ethanol precipitation and freeze dried. Equal amounts of mucus from each dog were pooled and stock suspension was prepared and stored at −20 °C until use.

Human and dog mucus were dissolved (0.5 mg/ml protein) in HEPES (*N*-2-hydroxyethylpiperazine-*N*-2-ethanosulphonic acid)-Hanks buffer (HH; 10 mmol HEPES, pH 7.4) and 100 μl of the solution was absorbed onto polystyrene microtitre plate wells (Maxisorp, Nunc, Denmark) by overnight incubation at 4 °C. Bacteria were metabolically labelled by the addition of 10 μl/ml ^{3}H-thymidine (Pharmacia/Amersham) to the MRS broth and incubated at 37 °C overnight. Radiolabelled bacteria were centrifuged (2000 × *g*, 7 min) and the pellet was washed (PBS, 5 mmol; pH 7.3) in order to standardize the number of bacteria approximatelly 10^{7} CFU/ml. Then bacteria (100 μl) were added into wells and incubated for at 37 °C, 1 h.

Table 1
Inhibitory activity of enterococcal isolates ($n = 40$)

Indicator strains	Source	Percentage of isolates with inhibitation zone			
		No zone	<6 mm	6–10 mm	>10 mm
Gram-positive					
E. avium EA 5	Faeces of piglet	35.0	7.5	22.5	35.0
E. casseliflavus 20332 TS	From collection[a]	80.0	12.5 (2.5)[b]	7.5	0.0
E. cecorum 266/7973	Dog[c]	27.5	7.5	10.0	55.0
E. durans 5A	Faeces of antelope	85.0	10.0 (10.0)	5.0 (5.0)	0.0
E. durans 266/S 118 4204	Puppy[c]	27.5	10.0	12.5	50.0
E. hirae 387266/8819	Cat[c]	85.0	7.5	7.5	0.0
E. faecalis EE P4	Faeces of Jap. quail	30.0	35.0 (2.5)	35.0	0.0
E. faecium EF 43	Faeces of piglet	27.5	17.5	40.0	15.0
L. acidophilus LA 99	Vegetable salad	45.0	30.0 (5.0)	22.5	2.5
L. johnsonii LJ 4982	Vegetable salad	87.5	10.0	2.5	0.0
Micrococcus sp. 4898	Fish salad	47.0	12.5 (5.0)	37.5	2.5
S. aureus SA 105	Mastitic milk	100.0	0.0	0.0	0.0
S. lentus SL 163	Faeces of deer	50.0	37.5 (35.0)	12.5	0.0
S. xylosus SX 310	Rumen cont. of calf	27.5	10.0 (5.0)	55.0	7.5
S. bovis AO 24/85	Rumen cont. of calf[d]	35.0	17.5	47.5	0.0
S.bovis SB 357	Pigeon[c]	50.0	17.5 (17.5)	32.5 (32.5)	0.0
S. lactis 96 RS	Unknown	42.5	25.0	32.5	0.0
Gram-negative					
E. coli W4	Faeces of dog	100.0	0.0	0.0	0.0
Enterobacter georgiviae EG3	pig slurry	100.0	0.0	0.0	0.0
Pseudomonas sp. E3	Faeces of dog	100.0	0.0	0.0	0.0
S. enterica subsp. *Enteritidis* SL 2/2	Clinical isolate[e]	100.0	0.0	0.0	0.0
Yersinia sp. M8	Faeces of dog	100.0	0.0	0.0	.0

[a] University Tuebingen, Germany.
[b] Percentage of isolates showing hazy zones of inhibition.
[c] University of Ghent, Belgium (Dr. L. Devriese).
[d] Institute of Experimantal Veterinary Medicine, Košice, Slovakia.
[e] Institute of Veterinary Medicine, Brno, Czek Republic (Dr. Šišák).

Subsequently, the wells were washed three times with 200 μl HH to remove unattached bacteria. The adherent bacteria were released and lysed with 1% SDS in 0.1 mol/l NaOH (200 μl per well) by incubation at 60 °C for 1 h. The radioactivity of the lysed bacterial suspension was measured by liquid scintillation. The adhesion ratio (%) was calculated by comparing the radioactivity of the bacteria added (triplicate 100 μl samples) to the radioactivity of the bound bacteria. The results shown are expressed as the average of at least three independent experiments in four parallel studies.

2.5. Antimicrobial activity

Antimicrobial activity was detected using the agar diffusion technique described by Skalka et al. (1983). Briefly, enterococci ($n = 40$) were inoculated on Brain Heart agar (Becton and Dickinson). The plates were incubated in an atmosphere 5% of CO_2 air at 37 °C for 24 h. Then, the plates were overlaid with 4 ml of appropriate soft agar (0.7 w/v%) inoculated with 200 μl of the overnight culture of indicator strain and incubated under the same conditions at 37 °C for 24 h. Inhibition was detected by a clear zone around the test organism. The indicator strains used in this study and their origin are listed in Table 1.

3. Results

Total mean counts of enterococci isolated from the faeces of 10 dogs ranged from 3.3 to 7.3 $\log_{10}$ CFU/g. Among 40 isolates, 11 were identified as *E. faecium*, two as *E. hirae* and further two belonged to the species *E. faecalis*. Twenty-five strains could not be speciated.

Thirty of 40 strains tested were found to produce bacteriocin-like inhibitory substances (BLIS; Table 1) with activity mainly against Gram-positive bacteria including the following genera: *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Micrococcus*, *Staphylococcus* and *Streptococcus*; however, not against Gram-negative organisms. Seven strains (*E. faecium* EF01, EF4, W1, *E. hirae* EH2, *E. faecalis* EE4, *Enterococcus* sp. E01, E05) with broad antimicrobial activity (Table 2) were selected for further detailed characterization. The broadest spectrum of antimicrobial activity was found by strain *E. faecalis* EE4. It inhibited all Gram-positive strains except *S. aureus* SA 105.

Table 2
Inhibitory activity of selected enterococcal strains against indicator microorganisms

Indicator strain	Strains tested for antimicrobial activity						
	E. hirae, EH2	*E. faecalis*, EE4	*E. faecium*, W1	*E.* sp., E01	*E.* sp., E05	*E. faecium*, EF01	*E. faecium*, EF4
E. avium EA 5	2	2	3	3	2	2	3
E. casseliflavus 20332 TS	1	2	–	–	–	1	–
E. cecorum 266/7973	1	3	3	3	3	1	3
E. durans 5A	1	2	–	–	–	–	–
E. durans 266/S 118 4204	1	2	3	3	1	1	3
E. hirae 387266/8819	–	2	–	–	–	1	–
E. faecalis EE P4	2	2	2	1	1	1	2
E. faecium EF 43	1	2	3	2	2	2	3
L. acidophilus LA 99	2	2	–	1	2	1	1
L. johnsonii LJ 4982	1	2	–	–	–	–	–
Micrococcus sp. 4898	1	2	2	2	–	2	2
S. aureus SA 105	–	–	–	–	–	–	–
S. lentus SL 163	2	2	1	1	1	–	1
S. xylosus SX 310	2	2	2	2	2	2	2
S. bovis AO 24/85	2	2	2	2	1	1	2
S. bovis SB 357	2	2	1	1	1	–	1
S. lactis 96 RS	2	2	1	1	1	1	2

'–': No inhibition; '1': inhibitioin zones smaller than 6 mm; '2': inhibition zones between 6 and 10 mm; '3': inhibition zones larger than 10 mm.

Table 3
Antibiotic resistrance of selected *Enterococcus* isolates

Antibiotic	Strain						
	E. faecalis, EH2	*E. faecium*, EF01	*E. faecium*, EF4	*Enterococcus* sp., E05	*Enterococcus* sp., E01	*E. faecium*, W1	*E. faecalis*, EE4
Ampicillin	S	S	S	S	S	S	S
Chloramphenicol	S	S	S	S	S	S	S
Erythromycin	S	S	S	S	S	S	S
Gentamicin	R	R	R	R	R	R	R
Kanamycin	R	R	R	R	R	R	R
Penicillin G	S	I	S	S	S	S	S
Tetracycline	S	S	S	S	S	S	S

R: resistant; S: sensitive, I: intermediate.

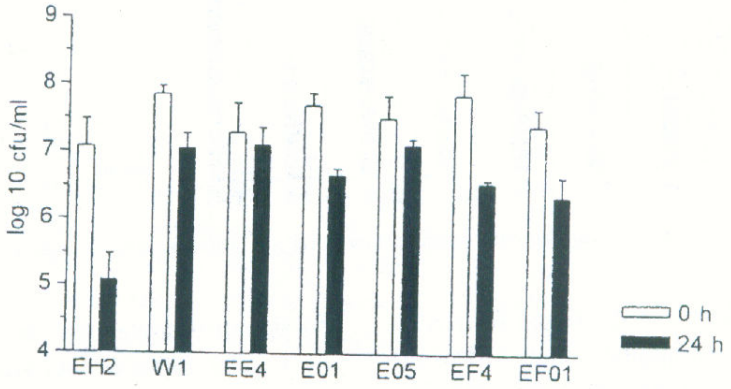

Fig. 1. Tolerance against bile of enterocci.

Among 40 isolates tested for antibiotic resistance, 13 (33%) exhibited resistance to erythromycin and 11 (28%) to tetracycline. All strains were sensitive to vancomycin, ampicillin, penicillin and chloramphenicol and resistant to kanamycin and gentamicin. The antibiotic character of seven selected strains is summarized in Table 3.

The survival rate of seven selected strains tested for their tolerance to 1% bile ranged between 72 and 98%. The best surviving strain in the presence of bile was *E. faecalis* EE4 (Fig. 1). Survival of strains at pH 3.0 was found to range between 76 and 87% after 3 h. *E. faecium* EF01 was found to have the best tolerance to low pH (Fig. 2). The adhesion to intestinal mucus

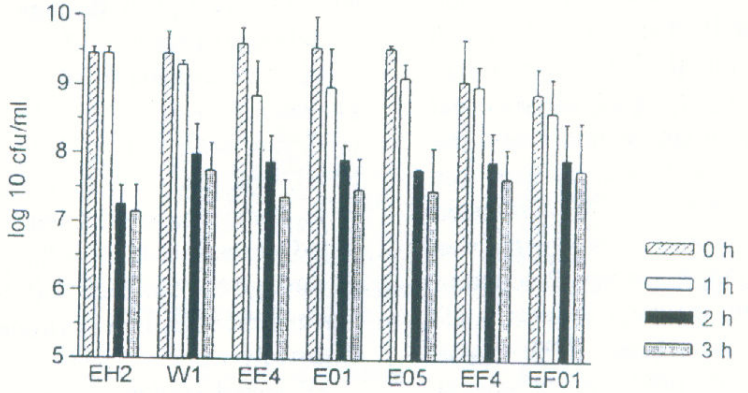

Fig. 2. Tolerance aganist pH 3.0 after 1–3 h of enterococcal strains.

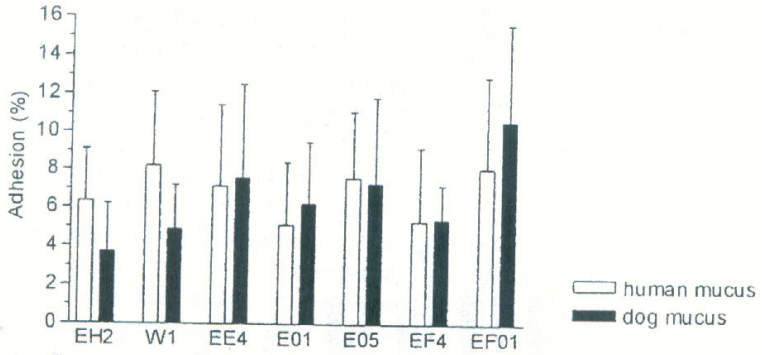

Fig. 3. The adhesion activity to human and dog intesstinal mucus of enterococci.

of the tested strains was found in the range from 4% (*E. faecium* EF01) to 11% for canine mucus and from 5% (*E. faecium* W1) to 8% for human mucus (Fig. 3). No significant difference was found in adherence to canine or human mucus.

4. Discussion

Enterococci are common inhabitants of animal intestine and they form the predominant microflora in the intestine during the first 2–3 days of life in many animals (Devriese et al., 1991). Zentek et al. (1998) found similar counts of enterococci in canine's ileum and faeces as is presented in this study. Although Devriese et al. (1992) identified *E. faecalis* and *E. hirae* as the most frequently isolated enterococcal species from anal swabs of dogs, in this study, the most common species among faecal isolates was found *E. faecium*. Most of the isolates (75%) produced BLIS. A wide range of Gram-positive organisms was inhibited, but none of the Gram-negatives. Production of BLIS by enterococci from different sources is already well documented (Lauková et al., 1993; Cintas et al., 1997). Many enterocins produced by several enterococci species have been purified and studied in detail (Cintas et al., 1997; Mareková et al., 2003). Bacteriocinogenic enterococci have been also isolated from the intestine (Du Toit et al., 2000). Enterocins generally belong to class II bacteriocins and have the potential to inhibit a wide spectrum of the organisms (Nes et al., 1996). Gram-negative bacteria are generally mostly resistant to bacteriocins from lactic acid bacteria because of their outer membrane providing a barrier to permeability. Nevertheless, several authors have also reported activity towards Gram-negative bacteria (Simonetta et al., 1997; Lauková et al., 2002). Bacteriocin-producing strains may play an important role in the maintenance of a desirable population in the gastrointestinal tract, i.e. by suppressing the growth of less desirable microbes.

Some authors consider antibiotic resistance beneficial in probiotic strains. Enterococci resistant to antibiotics can effectively protect the natural balance of intestinal microflora during and after therapy by the antibiotics to which they were proved resistant. Prior to reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach where the secretion of gastric acid represent a primary defense mechanism against the majority of ingested microorganisms. Among seven selected strains, three strains of *E. faecium* survived well at pH 3.0 after 3 h (over 85%), while the other four strains exhibited lower survival rates. The results indicated that several of the tested strains would have the potential to survive transit through the stomach and might possess the ability to reach the intestinal environment in which they may effectively work.

All tested strains survived in the presence of 1% bile under the rate over 85%. Tolerance to bile salts is a prerequisite for colonization and metabolic activity of probiotic bacteria in the small intestine of the host (Havenaar et al., 1992).

Ability of potential probiotic bacteria to adhere to the intestinal mucus is considered important for transient colonization, antagonism against pathogens, modulation of the immune system and enhanced healing of the damaged gastric mucosa (Alander et al.,

1999; Jin et al., 2000). The dog isolates were observed to bind to human mucus in a manner similar to that observed for canine mucus. The lack of species specificity was also obtained in earlier studies by Rinkinen et al. (2000) and Lauková et al. (in press).

Based on these results, *E. faecalis* EE4 and *E. faecium* EF01 have the prerequisites to survive in the gastrointestinal tract, to colonize transiently and to exert antimicrobial activity against target of Gram-positive bacteria and therefore, represent potential candidates for new probiotic strains. Further in vivo studies are warranted with these strains.

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