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Enterococci from Piglets – Probiotic Properties and Responsiveness to Natural Antibacterial Substances

V. STROMPFOVÁ, A. LAUKOVÁ

Institute of Animal Physiology, Slovak Academy of Sciences, 040 01 Košice, Slovakia
fax +421 557 287 842
e-mail strompfv@saske.sk

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ABSTRACT. Fifty-five strains of enterococci isolated from the piglet intestine were characterized *in vitro* for probiotic activity. Identification of the isolates revealed *Enterococcus faecium* as the predominant species (84 %). Forty strains (73 %) were found to produce bacteriocin-like substances (only into solid media) with activity almost only toward Gram-positive genera. Thirty-eight % of strains were resistant to tetracycline, 27 % to chloramphenicol, 18 % to erythromycin and 16 % to vancomycin. In addition to control of strain safety, 6 % of isolates were β-hemolytic and 16 % produced gelatinase. Seven strains selected for further probiotic assays exhibited sufficient survival rate at pH 3.0 after 3 h, in the presence of 1 % ox-bile and lysozyme after 1 d (over 10^7 CFU/mL in all tests). The adhesion of tested strains to porcine and human intestinal mucus was found in a similar range (1.4–14.0 % and 1.4–17.6 %, respectively). In accordance with current research effort to use and/or to combine various health promoting substances, the sensitivity of all isolates toward plant extracts and toward bacteriocins produced by animal and environmental strains was determined. All enterococci were sensitive toward oregano and sage extracts and toward one (*E. faecium* EF55 – chicken isolate, activity of 25 600 AU/mL) of ten bacteriocin substances. It means that a similar anti-enterococcal potential of some bacteriocin substances may be observed as for certain plant extracts.

Abbreviations

BHI	brain heart infusion (plate)	MRS	de Man–Rogosa–Sharpe (medium)
CFU	colony-forming unit	PPB	partially purified bacteriocin

Amp	ampicillin	Ery	erythromycin	Tet	tetracycline
Cmp	chloramphenicol	Rif	rifampicin	Van	vancomycin

Recently, the interest in healthy life together with the increasing problem of antibiotic resistance has made the alternative feed supplements increasingly popular. Industrial production of pigs without using antibiotics as growth promoters requires the combination of management and nutritional strategies, and alternative feed supplements, such as probiotics, prebiotics, herbal extracts, acidifiers, natural antimicrobial agents and antioxidants, minerals and/or enzymes (Stein 2007). However, currently there is no single substance, which could replace the function of feed antibiotics. Therefore, the search for appropriate combinations of different supplements could be an approach to achieve better performance (Bomba *et al*. 2006; Trojanová *et al*. 2006) in the use of less artificial additives.

Probiotics may constitute an effective and safe base for these combinations. Although the genus *Enterococcus* is the most controversial group of lactic acid bacteria (a lot of studies describe their positive and, *vice versa*, negative properties), some strains (*e.g*., of *E. faecium* and *E. faecalis* species) have been successfully used as probiotics because of their health-promoting ability (*European Commission* 2004). The improvement of growth performance, the reduction of post-weaning diarrhea modifying the immune response, and alteration of the intestinal microbiota were the major effects observed after application of probiotic enterococci to piglets (Pollmann *et al*. 2005; Scharek *et al*. 2005; Taras *et al*. 2006). Moreover, antimicrobial effects of certain probiotic strains could increase their capacity to produce antimicrobial peptides or proteins – bacteriocins (enterocins); these vary in the spectrum and mode of activity, molecular structure and molecular mass, thermostability, pH range of activity, and genetic determinats.

Enterocins are classified according to Franz *et al*. (2007) into class I bacteriocins – lantibiotic enterocins; class II enterocins – small, non-lantibiotic peptides including three subclasses; class III enterocins – cyclic antibacterial peptides, and class IV enterocins – large proteins. The large diversity of enterocins may reflect the robust and ubiquitous nature of enterococci, as well as their remarkable ability to disseminate and receive genetic material.

While the inhibitory effect of enterocins or enterocin-producers on the growth of food spoilage and pathogenic bacteria, such as *Listeria monocytogenes*, *Staphylococcus aureus*, *Bacillus subtilis*, *Clostridium perfringens*, *C. botulinum* in various food products and *in vitro* have been described (Lauková *et al*. 1999, 2001; Pantev *et al*. 2002), the information on bacteriocin production and their behavior in animal gastrointestinal tract is still scarce (Strompfová *et al*. 2003; Bernbom *et al*. 2006). Bacteriocins and/or bacteriocin-producing strains can reduce pathogenic or undesirable bacteria (*e.g*., *Salmonella* sp., *Campylobacter* sp., *Escherichia coli*) after their oral application to animals (Lauková *et al*. 2003; Ogunbanwo *et al*. 2004; Strompfová *et al*. 2006).

The aim of this study was to characterize enterococci isolated from the intestinal tract of healthy piglets as potentially new probiotics and to determine their sensitivity to bacteriocin substances as well as to plant extracts.

MATERIALS AND METHODS

Isolation and identification of enterococci. Rectal swab samples and feces collected from healthy sucklings and weaning piglets (n = 40, farms in Sečovce, Kysak, Salaška, Valaliky, Nižná Slaná, Veľká Ida, Kraľovce, Grajciar, Malá Ida, Slovakia; sampled in 1995–2001) were diluted in 0.85 % (*W/V*) NaCl and plated on M-*Enterococcus* agar (*Becton Dickinson*, USA). After incubation (2 d, 37 ºC), 55 colonies were picked-up and stored on M-*Enterococcus* agar for further testing. Species identification was performed by tRNA-intergenic length polymorphism analysis (tRNA-PCR; Baele *et al*. 2000) after DNA extraction from single colonies by alkaline lysis. PCR was done using the consensus primers T5A (5´-AGT CCG GTG CTC TAA CCA -AC TGA G-3´) and T3B (5´-AGG TCG CGG GTT CGA ATC C-3´; Welsh and McClelland 1991). PCR products were analyzed by capillary electrophoresis and interpreted according to Baele *et al*. (2000). Twenty isolates were identified to species level using the API 20 Strep kit (*bioMérieux*, France).

Sensitivity and/or resistance toward antimicrobials, low pH, bile and lysozyme. Antimicrobial sensitivity and/or resistance was tested by the agar-diffusion method using the following disks (*Becton Dickinson*): Cmp, Tet, Van, Rif (all 30 μg), Ery (15 μg), Amp (10 μg). *E. faecium* CCM 4231 was used as the control strain. Resistance to bile was tested according to Gilliland and Walker (1990). Overnight cultures of the tested strains were inoculated (2 %) into MRS broth (*Merck*, Germany) with and without the addition of 1 % (*W/V*) purified ox-bile (*Biomark*, India) and incubated for 1 d at 37 °C. Before and after incubation, samples were plated on M-*Enterococcus* agar. To test survival of the isolates at low pH, the cells of overnight cultures (MRS broth) were harvested by centrifugation (2000 ***g***, 15 min), resuspended in 0.05 mol/L phosphate buffer (pH 3), and kept for 3 h at 37 °C. CFU were determined on M-*Enterococcus* agar. Resistance to lysozyme was detected in BHI broth (*Becton Dickinson*) with and without lysozyme (100 μg/mL). The broth was inoculated (2 %) by overnight cultures and incubated for 1 d at 37 °C. Samples were plated on BHI agar plates at the start and after a 1-d incubation.

Mucus adhesion assay. Adhesion to porcine and human intestinal mucus was studied according to Ouwehand *et al*. (1999) in polystyrene microtitre plate wells (*Maxisorp*, Denmark) with immobilized mucus. Mucus was isolated from the healthy part of resected colonic tissue and treated according to Ouwehand *et al*. (2002). Labeled bacteria (methyl-1,2-^{3}H-thymidine; *Amersham International*, UK) were added into the wells and incubated for 1 h at 37 °C. After removing unattached bacteria, the radioactivity of adherent (lysed with 1 % SDS) bacteria was measured by liquid scintillation. The adhesion (in %) was calculated by comparing the radioactivity of the bacteria added to the radioactivity of the bound bacteria.

Detection of gelatinase and hemolytic activity. Gelatinase production was detected on BHI agar (*Becton Dickinson*) supplemented with gelatinase (30 g/L; Drahovská *et al*. 2004). Blood hemolysis was evaluated on Columbia agar plates (*Becton Dickinson*) supplemented with 5 % of sheep blood, incubated for 1 d at 37 °C.

Antimicrobial activity of enterococci was tested by the agar-diffusion technique (Skalka *et al*. 1983) on phosphate-buffered BHI plates. Inhibition was detected by a clear or hazy zone around the test organism (for indicator strains *see* Table I).

Sensitivity of strains toward bacteriocin substances and plant extracts. Susceptibility of enterococci to oregano extract, sage extract and to PPB of 10 strains (our isolates, except for *E. haemoperoxidus* which was provided by Dr. I. Sedláček, *Masaryk University*, Brno, Czechia) was tested by the agar-spot method (De Vuyst *et al*. 1996) on BHI agar plates. The *Salvia officinalis* (24 % thujone, 18 % borneol, 15 % cineole) and *Origanum vulgare* extracts (55 % carvacrol; both from *Calendula a.s.*, Nová Ľubovňa, Slovakia) were provided by Assoc.Prof. Šalamon and Assoc.Prof. Poráčová (*University of Prešov*, Slovakia).

Table I. Inhibitory spectrum of enterococci isolated from piglets (n = 55)

Indicator strain	Source	Number of isolates with inhibition zone		
		no zone	6–12 mm	>12 mm
Gram-positive bacteria				
Enterococcus avium EA5	feces of piglet	16	6	33
E. casseliflavus 20332 TS	from collection[a]	49	6	0
E. durans 5A	feces of antelope	51	4	0
E. faecalis EE P4	feces of Japanese quail	16	6 (6)[f]	33 (32)[f]
E. faecium EF 43	feces of piglet	15	6	34
Lactobacillus acidophilus LA 99	vegetable salad	17	3	35 (35)[f]
L. johnsonii LJ 4982	vegetable salad	53	2	0
Lactococcus lactis 96RS	from collection[b]	19	4	32
Listeria monocytogenes CCM 4699	from collection[c]	16	6	33
Micrococcus sp. 4898	fish salad	16	5	34
Staphylococcus aureus SA2	fish salad	17	5	33
S. lentus SL 163	feces of deer	18	18 (17)[f]	19 (19)[f]
S. xylosus SX 310	rumen content of calf	19	3	33
S. aureus SA 2	mastitic milk	18	6 (3)[f]	31
S. aureus SA 105	mastitic milk	55	0	0
Streptococcus bovis AO 24/85	rumen content of calf[d]	21	7 (4)[f]	27 (27)[f]
S. bovis SB 357	pigeon[b]	21	7	27
Gram-negative bacteria				
Escherichia coli W4	feces of dog	55	0	0
Enterobacter georgiviae EG3	pig slurry	54	1 (1)[f]	0
Pseudomonas sp. E3	feces of dog	54	1 (1)[f]	0
Salmonella enterica sv. Enteritidis SL 2/2	clinical isolate[e]	55	0	0
Yersinia sp. M8	feces of dog	55	0	0

[a]*Universität Tübingen*, Germany. [b]*University of Ghent*, Belgium. [c]*Czech Collection of Microorganisms*, Brno, Czech Republic.
[d]*University of Veterinary Medicine, Research Institute of Gnotobiology and Diseases in Young*, Košice, Slovakia.
[e]*Institute of Veterinary Medicine*, Brno, Czech Republic.
[f]Number of isolates (in *parentheses*) exhibiting hazy zones of inhibition.

The PPBs (produced by strains listed in Table III) were prepared by the following procedure: a 16-h culture (300 mL) of strain in MRS broth (*Merck*) was centrifuged (10000 ***g***, 30 min) in order to remove the cells. After adjusting of supernatant to pH 5.0, diammonium sulfate was gently added to the supernatant (40 %, *W/V*, saturation), and the mixture was stirred for 1 d at 4 °C, or for 1 h at 21 °C depending on individual strain. After centrifugation (10000 ***g***, 30 min), the resulting pellet was resuspended in 10 mmol/L sodium phosphate buffer (pH 6.5). *E. avium* EA5 (our piglet isolate) was used as a bacteriocin-sensitive indicator to determine bacteriocin activity levels.

RESULTS

Out of total of 55 enterococcal strains, *E. faecium* (37 strains, 67.3 %) was the most predominant species followed by *E. faecalis* (4 strains, 7.3 %), *E. casseliflavus* (2 strains, 3.6 %), and *E. avium* (1 strain, 1.8 %; 11 strains were not taxonomically assigned).

Determination of antimicrobial profiles revealed 38 % resistance to Tet (21 resistant strains), 27 % to Cmp (15 strains), 18 % to Ery (10 strains), 16 % to Van (9 strains) and only 2 % resistance to Rif (1 strain). All strains were Amp-sensitive. Twenty-eight strains were sensitive to all tested antimicrobials. Taking into account the period of isolation, 25.5 % of strains isolated in 1995 were sensitive to tested antimicrobials compared to 65.7 % of sensitive strains isolated in 2001.

Forty strains (73 %; 27 *E. faecium*, 2 *E. faecalis*, 2 *E. casseliflavus*, 9 *Enterococcus* sp.) were found to produce bacteriocin-like substances with activity almost only against Gram-positive bacteria (Table I). The most sensitive indicator bacterium was *E. faecium* EF43 (piglet isolate). The broadest spectrum of antimicrobial activity was observed for the strain *Enterococcus* sp. C62. It inhibited all tested Gram-positive strains (except *S. aureus* SA105) and partially also Gram-negative strains (hazy zones of inhibiton). Among

bacteriocin-producing enterococci, all strains were able to produce bacteriocin-like substance only into solid agar media. None of the strains inhibited the same indicator bacteria by agar spot test using neutralized culture supernatant.

Only 3 strains (5.5 %; 2 *E. faecalis*, 1 *Enterococcus* sp.) showed β-hemolysis on blood agar and 9 strains (16.4 %; 3 *E. faecalis*, 6 *Enterococcus* sp.) exhibited gelatinase activity (positive strains were excluded from further assays).

Seven bacteriocin-producing strains were selected for detection of their tolerance to bile, low pH and lysozyme (Table II). The survival in the presence of 1 % *W/V* ox-bile after a 1-d incubation was observed between 7.9 and 8.7 $\log_{10}$ CFU/mL (reduction by 0.6–1.1 log cycle). The viable counts of the strains at pH 3.0 were stable or decreased only slowly for the first hour of incubation (reduced by 0.1–1.1 log cycle), followed by a greater reduction at the end of the 3-h incubation period (by 1.5–2.3 log cycle). In the presence of lysozyme, no reduction in viable counts for any of the five *E. faecium* strains was observed. Only C21 and C62 strains were less resistant to lysozyme and their counts were decreased by 0.8–0.9 log cycle after 1 d. The adhesion of the tested strains to intestinal mucus was found in the range of 1.4–14.0 % for porcine and 1.4–17.6 % for human mucus (3 strains showed higher adhesive capacity to human than to porcine intestinal mucus).

All enterococci (n = 55) were tested for their sensitivity to bacteriocin substances produced by *E. faecium* and *E. haemoperoxidus* strains (Table III). The PPS of *E. faecium* EF55 with activity of 25600 AU/mL was the most effective substance in inhibiting the growth of piglet strains. The inhibitory effect was shown to be dependent on initial activity of substances. The exception is the PPS of *E. haemoperoxidus* species which was less potent despite the 12800 AU/mL activity against the principal indicator. The plant extracts of oregano and sage also included in this screening assay inhibited the growth of all tested enterococci (Table III).

DISCUSSION

Of a variety of isolated porcine intestinal enterococci *E. faecium* seems, according to our results, to be the major cultivable enterococcal species in pigs, followed by *E. faecalis*, which agrees also with species composition detected in pork meat (Kročko *et al*. 2007).

The highly efficient ability of enterococci to transfer antibiotic-resistance genes makes the examination of this safety feature a very important factor. The resistance to Van was found to be more frequent comparing with isolates from dogs or chickens (Strompfová *et al.* 2004). The favorable result is the reduction of Van-resistant strains in the time (6 strains in 1995, 3 strains only in 2001). The higher resistance of piglet enterococci to Tet could be expected since Tet is still frequently being used in animal breeding. Very high resistance rates toward Tet were detected by Butaye *et al.* (2001) (100 % resistant enterococci from pigs) and by Šustáčková *et al.* (2004) (35 % resistant isolates from raw beef and meat products). The use of Cmp in animal husbandry is banned in Europe; despite this, we found 27 % of resistant strains.

Generally, the evaluation of virulence factors is necessary for selecting safe probiotic strains. Hemolytic activity was detected only in 6 % of strains from piglets compared with 11–70 % in clinical isolates or 0–25 % in fecal isolates (Eaton and Gasson 2001; Drahovská *et al.* 2004). Gelatinase (extracellular metalloendopeptidase hydrolyzing gelatin, collagen and hemoglobin) was produced by 16 % of enterococcal isolates in contrast to the high occurrence (55–100 %) in clinical isolates in other studies (Eaton and Gasson 2001).

Bacteriocin-producing enterococci seem not to prefer any special niches. Seventy-two % of tested enterococci exhibited bacteriocin-like activity. Likewise, a high occurrence of ability to produce bacteriocin-like substances among enterococci of various origin was observed, *e.g*., in 70 % of ruminant isolates (Lauková and Mareková 2001) or 75 % of canine isolates (Strompfová *et al*. 2004). However, enterococci from piglets showed detectable inhibitory activity only on a solid medium (similarly as canine fecal isolates). The apparent inability to produce the bacteriocin substances in liquid media is not rare (Fricourt *et al*. 1994) and may reflect the fact that the organism exists in contact with solids when in nature. Ninety-eight % of bacteriocinogenic isolates were active against *Listeria* sp. which corresponds to a strong antilisterial effect of most enterocins known. Such property, together with heat stability, led to a wide application of enterocins as biopreservatives in food systems. The detection of enterocin structural genes in enterococci from piglets (*see* Strompfová *et al.* 2008) revealed that almost 60 % of strains possess one or more enterocin gene(s) among tested enterocin A, P, B, L50B genes. The gene of enterocin P was the most frequent, followed by gene of the L50B enterocin.

Table II. Resistance of selected strains to bile and low pH (log_{10} CFU/mL)[a]

Strain	Bile (1 %)		pH 3.0				Lysozyme (100 µg/mL)	
	0 h	24 h	0 h	1 h	2 h	3 h	0 h	24 h
E. faecium O52	9.05 ± 0.07	8.43 ± 0.16	9.63 ± 0.25	9.09 ± 0.77	8.69 ± 0.24	7.86 ± 0.06	8.90 ± 0.05	10.14 ± 0.11
E. faecium O34	9.35 ± 0.09	8.43 ± 0.30	9.79 ± 0.13	9.17 ± 0.60	8.59 ± 0.36	7.54 ± 0.51	8.85 ± 0.15	9.62 ± 0.11
E. faecium O12	9.14 ± 0.17	8.49 ± 0.06	9.61 ± 0.18	9.69 ± 0.42	8.92 ± 0.04	7.82 ± 0.18	9.16 ± 0.14	9.90 ± 0.06
E. faecium C44	9.42 ± 0.13	8.60 ± 0.07	9.58 ± 0.38	9.16 ± 0.07	8.88 ± 0.26	7.96 ± 0.04	8.93 ± 0.38	9.69 ± 0.04
E. faecium O64	9.19 ± 0.07	8.55 ± 0.12	9.48 ± 0.37	9.04 ± 0.33	8.65 ± 0.21	8.01 ± 0.14	8.98 ± 0.20	9.48 ± 0.30
E. faecalis C21	9.00 ± 0.23	7.90 ± 0.19	9.80 ± 0.16	8.67 ± 0.37	8.21 ± 0.26	7.83 ± 0.36	9.08 ± 0.01	8.15 ± 0.05
Enterococcus sp. C62	9.49 ± 0.37	8.74 ± 0.21	9.82 ± 0.24	9.71 ± 0.34	8.99 ± 0.16	8.37 ± 0.28	9.04 ± 0.26	8.29 ± 0.08

[a]Means ±SD; for further details *see* text.

Table III. Sensitivity of enterococci from piglets to partially purified bacteriocins and plant extracts

PPB	Origin	Activity[a] AU/mL	Inhibited		Partially inhibited[b]		Resistant, number
			number	zone[c], mm	number	zone[c], mm	
Enterococci from piglets ($n = 55$)							
E. faecium EF55	crop of chicken	25 600	51	20.0	4	15.5	0
E. faecium EF2019	feces of rabbit	25 600	29	13.0	17	14.0	9
E. faecium EF9296	silage	12 800	18	12.0	29	13.0	8
E. faecium CCM7419 (*ent*A, P)	cattle dung water	12 800	11	16.0	32	13.5	12
E. haemoperoxidus CCM466[d]	surface water	12 800	0	0.0	8	10.0	47
E. faecium EF24/10	feces of rabbit	6400	17	11.0	14	10.5	24
E. faecium AL41 (*ent* M)	cattle dung water	6400	10	12.5	17	12.0	28
E. faecium EF2119	feces of rabbit	3200	1	10.0	9	10.0	45
E. faecium EF1819	feces of rabbit	400	14	10.0	23	13.0	18
E. faecium CCM4231	rumen content of calf	100	1	10.0	3	8.0	51
Phytoadditives							
Origanum vulgare extract	both *Calendula a.s.*,	–	55	30.0	0	0	0
Salvia officinalis extract	Nová Ľubovňa, Slovakia	–	53	10.0	2	10.5	0

[a]*E. faecium* strain EA5 was used as indicator. [b]Hazy zone of inhibition. [c]Median. [d]Provided by *Czech Culture Collection*, Brno, Czechia.

Seven selected bacteriocin-producing strains were capable of surviving the unfavorable pH 3 of gastric environment and the presence of 1 % ox-bile; their viable cell counts remained $>10^7$ CFU/mL (sufficient for *in situ* activity in the intestine). *E. faecalis* was more sensitive to bile and low pH than *E. faecium*, which could explain why the counts of this species are lower in pig intestine. This indicates that the survival of bacteria in bile environment as well as at low pH is a species- and also origin-dependent property (*cf*. Park *et al*. 2006). Generally, the strains of gastrointestinal origin are more tolerant than the strains of food or environmental origin. Growth of *E. faecium* was not affected by the presence of lysozyme (negative effector for bacteria in oral cavity and intestinal tract). Higher sensitivity to lysozyme was observed again for *E. faecalis* strain, but its counts remained $>10^8$ CFU/mL.

Adhesion tests to intestinal mucus revealed sufficient but not strong adhesion to porcine intestinal mucus (1.4–14.0 %). Similar or higher (in 3 strains) level of adhesion was observed for human mucus which confirmed earlier observations that there is a rather low specificity in adhesion of lactic acid bacteria to intestinal mucus from various hosts under *in vitro* conditions (Lauková *et al.* 2004).

The efficiency of probiotics can be enhanced by their combination with plants. Antimicrobial activity of some plant extracts has been described with a distinct level of selectivity in these effects (Si *et al.* 2006). Oregano, in particular, has a potential to replace sub-therapeutic doses of antibiotics in pig feed. We showed that oregano extract as well as sage extract inhibit the growth of all tested enterococci. Chemical composition of tested essential oils plays an important role in their antimicrobial activity. For example, phenols, such as carvacrol (isoterpenoid phenol, main active component of oregano), generally show a strong antimicrobial activity and exhibit some level of selectivity toward pathogens with little effect on lactobacilli and/or anerobic bacteria in the digesta (Si *et al.* 2006). We observed that bacteriocin substances possess similar antimicrobial effect as the plant extracts used, at least toward piglet enterococci (*e.g.*, PPB of *E. faecium* EF55 inhibited all strains).

The ecosystem in gastrointestinal tract of healthy piglets may serve as a potential source for the isolation of potential probiotic and/or bacteriocin-producing basteria, which survive better under these less favorable conditions. Sensitivity of piglet enterococci to plant extracts and bacteriocins at certain concentrations should be taken into account in developing the optimum combination of components used in the replacement of antibiotics. These antimicrobial substances should, however, be applied before the administration of probiotic enterococci, in contrast to synergistically acting components that are usually combined with probiotics (*e.g.*, oligosaccharides, 'omega-3 polyunsaturated fatty acids').

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