Enterococci from rabbits – potential feed additive

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ABSTRACT: Enterococci (58) from faeces of rabbits of various age (from 2 months to 3 years) and 5 different rabbit farms were isolated and tested for survival in the presence of oxgall, lactic acid production, urease activity, resistance to low pH as well as their binding ability was tested. Fifty percent of enterococcal isolates were identified as *Enterococcus faecium*, 19% as *E. faecalis*. All strains showed good survival in the presence of 5% oxgall. The urease activity of isolates was in the range from 0.013 to 17.13 nkat/ml, only *E. faecalis* EE229 strain did not produce any urease. The survival of strains was tested at pH 3.0 and the percentage of their survival ranged between 62.0% and 90.0%. *E. faecium* EF1819 strain was found to show the best survival ability at low pH. Particle agglutination assay values of selected enterococci expressed only negative (0) or weakly positive (1) binding of heparin, bovine fibrinogen, porcine fibronectin and lactoferrin. Based on the results, most of the selected enterococci could be promising probiotic feed additives.

Keywords: probiotic feed additive; rabbit; enterococci; properties

Nowadays, probiotics are used in veterinary and human medicine as well as in nutrition with increasing frequency. Probiotics are defined as direct feed microbials or microbial cell preparations with a beneficial effect on the health and well-being of the host (Fuller et al., 1989; Nemcová et al., 1997). In contrast to human nutrition, where lactobacilli are predominant probiotic products, in animal nutrition *Enterococcus* sp. as well as yeasts from the genus *Saccharomyces* are the most frequently used as probiotic microorganisms (Simon et al., 2001). Nowadays, rabbit breeding has an increasing impact due to the nutritional value of meat and economic traits of breeding. Development in this animal industry also induces to search for new technologies to improve rabbit health and welfare. The improvement of rabbit health and performance by addition of probiotics and organic acids to feed mixtures was already studied (Skřivanová and Marounek, 2002). However, an alternative could also be the application of probiotic microorganisms, especially those isolated from the intestinal ecosystem of rab-

bits. The rabbit caecum is predominantly colonized by strict anaerobes such as members of the genus *Bacteroides* within the first weeks after birth. The composition of caecal microflora changes with advancing age (Gouet and Fonty, 1979). However, the presence of facultative anaerobic bacteria such as enterococci is also important because enterococci colonize rabbits' digestive tract and belong to the lactic acid bacteria (LAB) which are frequently of probiotic character. A potentially successful probiotic strain is expected to have several desirable properties such as strain origin, acid and bile tolerance, adherence to the intestinal epithelium and colonization ability, production of antimicrobial substances as well as good technological properties (Salminen et al., 1998). In addition, ureolytic activity is one of the most important metabolic properties especially concerning the enterococci.

The purpose of this study was to select enterococci from the digestive tract of rabbits and to test their metabolic properties with the aim to select a suitable probiotic feed additive.

MATERIAL AND METHODS

Isolation of enterococci. Samples were collected from 23 rabbits from five different rabbit farms. Among 23 animals, 6 were New Zealand rabbits, 8 were Californian growing rabbits and the others (9) were crossbred rabbits of various age (from 2 months to 3 years) as follows: 2 months old – 1 rabbit, 4 months old – 1 rabbit, 5 months old – 3 rabbits, 7 months old – 2 rabbits, 12 months old – 7 rabbits, 15 months old – 4 rabbits, 24 months old – 4 rabbits and 36 months old – 1 rabbit). Rabbits on one farm were kept indoors in flat deck batteries while rabbits on the other farms were housed in semi-open flat deck batteries. All animals were fed commercial granulated feed (Norm Typ, BIOFER s.r.o. Prešov, Slovak Republic). Additionally, they also received wheat, barley, oats and hay *ad libitum*. To isolate enterococci the samples of faeces were diluted by a standard microbiological method in (0.85%) saline buffer. The appropriate dilutions were plated onto M-Enterococcus agar (ME, Becton and Dickinson, Cockeysville, USA) and incubated at 37°C for 24 h in partially CO_2/air atmosphere. After incubation, the colony forming units (CFU) were counted. Then, 58 colonies of enterococci (including each samples) were randomly picked up and maintained on ME agar for further identification and testing. Isolates of enterococci were phenotyped by BBL Crystal ID kits (Becton and Dickinson) and genotyped by PCR followed by agarose electrophoresis according to Woodford et al. (1997).

Resistance to bile. Resistance to bile was tested according to Gilliland and Walker (1990). Brain Heart Infusion broth (BHI, Becton and Dickinson) was prepared by the addition of 5% (w/v) of oxgall (Becton and Dickinson). The volume 50 µl of an 18-hour culture of each strain was added to 5 ml of BHI broth with oxgall. After incubation at 37°C for 24 h, the bacterial growth of strains was measured spectrophotometrically at 600 nm. Numbers of viable cells were estimated at 0 h and after 24 h of incubation using M-Enterococcus agar. *Enterococcus faecium* CCM 4231 was used as a positive control (Lauková et al., 1993).

Urease activity. The method according to Cook (1976) was used to examine the urease activity. It was expressed in nkat/ml.

Resistance to low pH. To test the survival of isolates at low pH values, the cells of overnight cultures in BHI (Becton and Dickinson) of selected strains were harvested by centrifugation (2 000 × g for 15 min), resuspended in 0.05 M phosphate buffer of pH 3.0 adjusted with 1 N HCl and kept at 37°C for 1, 2 and 3 h. The CFU were determined on ME agar (Becton and Dickinson).

Particle agglutination assay (PAA). The protein-coated latex beads (20 µl) were mixed on a glass slide with an equal volume of a bacterial cell suspension of 10^{10} CFU/ml. These two drops were gently mixed and the agglutination reaction was scored after 2 min as a PAA value from strongly positive (3) to negative (0) as was previously described by Naidu et al. (1998) and Štyriak et al. (1999).

RESULTS

The total counts of enterococci from faeces of rabbits ranged from 10^3 to 10^5 CFU/ml/g. Among 58 isolates specified by PCR, 29 strains were classified as *E. faecium* and 11 strains belonged to
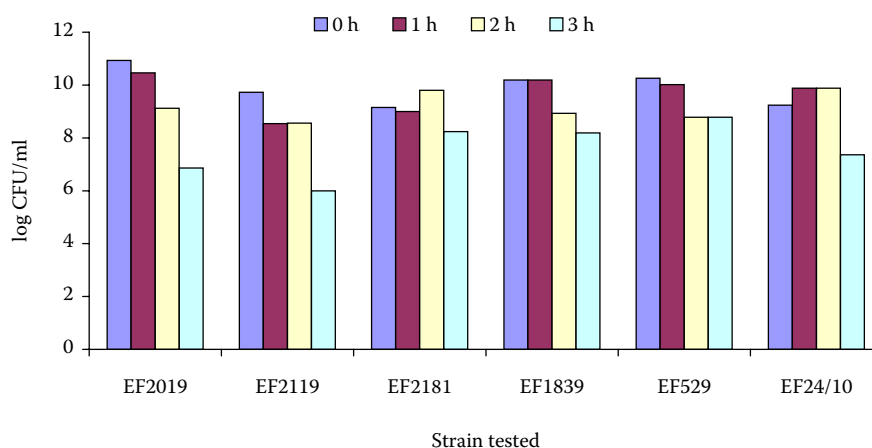

Figure 1. Survival of enterococci incubated at pH 3.0 for 0, 1, 2 and 3 h

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TablE1. Urease activity of enterococci

Isolates	Urease (nkat/ml ± SD)	Isolates	Urease (nkat/ml ± SD)
EF1119/23	2.55 ± 0.81	EF419/23	8.72 ± 2.25
EF1129/23	0.98 ± 0.00	EF519/23	2.39 ± 0.44
EF1179/23	1.03 ± 0.32	EF1129/23	13.07 ± 3.76
EF69/23	2.29 ±1.14	EF2139/23	3.95 ± 1.76
11K/1	2.12 ± 1.68	EF929/23	6.31 ± 4.47
EF329/23	1.09 ± 0.11	EF529/23	4.39 ± 1.76
EF429/23	1.78 ± 0.14	EF729/23	0.50 ± 0.17
EF739/23	2.70 ± 0.58	EF629/23	1.27 ± 0.48
EF819/23	3.47 ± 0.10	EF839/23	6.95 ± 0.37
EF939/23	3.68 ± 2.71	EF1839/23	3.95 ± 0.44
EF1819/23	5,21 ± 3,13	EF24/10	12.50 ± 1.87
EF2019/23	0.73 ± 0.55	EF349/23	17.13 ± 2.25
EF2119/23	2.59 ± 0.09	EF639/23	6.40 ± 1.55
EF219/23	4.78 ± 2.10	EF659/23	2.68 ± 0.30
EF319/23	9.11 ± 1.81	EF2129/23	1.04 ± 0.74
EF1219	2.08 ± 0.00	Esp1829	2.20 ± 0.04
EE829	3.87 ± 0.30	Esp1639	1.31 ± 0.07
EE1229	3.13 ± 0.75	Esp1239	1.55 ± 0.04
EE259	8.78 ± 1.34	Esp1339	1.18 ± 0.59
EE339	3.72 ± 0.45	Esp539	1.59 ± 0.21
EE1529	2.83 ± 0.75	Esp249	0.28 ± 0.07
EE2319	3.72 ± 1.04	Esp649	0.45 ± 0.24
EE1539	0.32 ± 0.07	Esp1429	0.55 ± 0.21
EE719	1.65 ± 0.62	Esp2339	0.66 ± 0.04
EE549	1.21 ± 0.45	Esp239	2.21 ± 0.76
EE229	0 ± 0	Esp1519	0.48 ± 0.14
EE1629	0.013 ± 0.00	Esp1619	0.97 ± 0.14
Esp2329	0.89 ± 0.35	Esp1319	0.83 ± 0.41
Esp1329	2.14 ± 0.90	Esp1419	0.21 ± 0.00

E. faecalis. Strains of *E. faecalis* were firstly phenotypically characterized by BBL Crystal ID kits (Becton and Dickinson) and confirmed by PCR method.

Enterococci were able to reach 10^8–10^9 CFU/ml in the presence of oxgall in comparison with the control growth (10^{11}–10^{12} CFU/ml) in oxgall-free broth.

The ureolytic activity of enterococcal isolates was detected in the range from 0.013 ± 0.0 to 17.13 ± 2.25 nkat/ml (Table 1). Fourteen strains of enterococci showed low ureolytic activity (0.013 ± 0.0 and 0.98 ± 0.0 nkat/ml) and in 9 strains the highest values of ureolytic activity were measured (in the range from 6.31 ± 2.47 to 17.134 ± 2.25 nkat/ml). The most of strains (37) were detected with mean

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Table 2. Binding of extracellular matrix molecules by strains with bacteriocinogenic activity tested by particle agglutination assay (PAA)

Isolates	HEP	BFG	BFN	BL
EF2119	0	0	0	1
EF2019	0	0	0	0
EF1819	0	0	0	0
EF24/10	0	0	0	1
EF1839	0	0	0	0
EF529	0	0	0	0

HEP – heparin, BFG – bovine fibrinogen, BFN – bov. fibronectin, BL – bov. lactoferrin, EF – *E. faecium*

values of urease expression (1.03 ± 0.32 and 5.21 ± 3.13 nkat/ml). The strain *E. faecalis* EE229 did not produce any urease.

Six bacteriocin-producing strains (Simonová and Lauková, 2004a) showed good resistance to low pH. As for the survival of tested strains at pH 3.0, it ranged between 62.0 and 90.0% (Figure 1). *E. faecium* EF1819 strain showed the best ability to survive at low pH (90.0%).

PAA values of 6 selected enterococcal strains from rabbits expressed only the negative (0) binding of heparin (HEP), bovine fibrinogen (BFIB) and porcine fibronectin (PFIB, Table 2). The binding of bovine lactoferrin (BLACT) was negative in four *E. faecium* strains (EF1839, EF529, EF2019, EF1819) and weakly positive (1) in *E. faecium* EF24/10 and EF2119.

DISCUSSION

Enterococci are common inhabitants of animal intestines and they are the predominant intestinal microflora during the first 2–3 days of life of many animals (DeVriese et al., 1991; Lauková, 1996). The majority of studies deals with the beneficial effect of probiotic enterococci and/or lactobacilli in poultry, pigs or dogs (Bomba et al., 1996; Lauková et al., 2003; Strompfová et al., 2004), but not in rabbits. However, lactobacilli are very scarcely found in rabbits' gastrointestinal tract (Yu and Tsen, 1993). Moreover, until now only little information has been provided concerning the probiotic utilization in rabbits and no strain originated from the rabbit ecosystem (Simonová and Lauková, 2004b).

For this reason the attention is focused on enterococcal strains from rabbits which are present in sufficient numbers there and which are also able to produce antimicrobial substances. In our study, twenty-nine isolates (50%) were identified as *E. faecium*. On the other hand, Linaje et al. (2004) described the prevalence of *E. faecalis* (58% out of 24 strains). Only 34% was identified as *E. faecium* and 8% as *E. durans* isolated from both the faeces and the caecum content of rabbits. The survival ability of enterococcal strains in 4% bile was also reported by Linaje et al. (2004); which is in accordance with our results. These authors also reported good resistance of enterococci to an acid environment (survival after 4 h of incubation in an acid medium). This fact indicates high bile resistance and good survival possibilities of enterococci in the digestive tract of animals in general (Marciňáková et al., 2004).

The rabbit caecal microflora is characterized by several enzymatic activities, e.g. cellulolytic, pectinolytic, amylolytic, proteolytic and ureolytic ones (Gidenne, 1997). In general, urease is an important enzyme from the clinical aspect (to determine urea in blood) as well as in nitrogen metabolism in ruminants (Hausinger, 1986). Moreover, the ureolytic activity of enterococci is an important process from the viewpoint of the basic metabolic properties. In our study, enterococci isolated from rabbits produced lower levels of urease compared to the ureolytic activity of ruminal enterococci (Lauková and Juriš, 1997).

Among the criteria for strains that could be used as probiotic feed additive their ability to colonize the mucosal surfaces and to prevent the attachment of pathogens is also included. Enterococcal strains isolated from silage and commercially known probiotic strain e.g. *E. faecium* M74 expressed the similar binding activity as was detected in our isolates (Štyriak et al., 2003). Perhaps the capacity of enterococci to bind bovine lactoferrin – iron-binding protein – is correlated with their growth or virulence (Schryvers and Gonzales, 1989). In comparison with the binding of enterococci from rabbits described previously by Štyriak et al. (2004), our isolates possessed the same or lower binding ability with lactoferrin, and also with other extracellular matrix (ECM)-binding proteins. The reason for their lower binding ability with lactoferrin could be explained by their origin from healthy animals without any clinical signs or disease.

Based on the results, a majority of the studied enterococci possessed "probiotic" properties. The isolates were characterized by good capability to survive in the presence of oxgall and low pH; most of them showed the ureolytic activity, ability to produce lactic acid. Moreover, as previously described by Simonová and Lauková (2004a) the isolates of *E. faecium* from rabbits also possessed bacteriocinogenic properties. It can be concluded that enterococci with performed characteristics could be selected for further utilization as probiotic feed additives. Of course, further experimental applications are needed.

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