

Full Length Research Paper

Isolation and characterization of potential probiotic enterococci strains from soft cheese flora

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Received 2 January, 2017; Accepted 7 March, 2017

This study was conducted to determine the *in-vitro* probiotic properties of *Enterococcus faecium* strains isolated from soft cheese. To evaluate the safety of *Enterococcus* strains, we compared the pathogenic genes, antimicrobial susceptibility of the probiotic strains to those of clinical isolates, and their antimicrobial activity against food-borne pathogenic and spoilage bacteria. *Enterococcus* strains were identified and evaluated *in vitro* for biochemistry methods acid, bile salts, lysozyme and pancreatin tolerance. One hundred and three strains were identified as *E. faecium*, and none of them were no vancomycin-resistant, and no pathogenic genes – such as *cylA*, *asa1*, *gelE*, *ace* and *cpd* – were found. The isolates showed good viability at 120 and 240 min of incubation with pH 3.0, and were able to resist 0.3% and 0.1 g/ml of bile salts and pancreatic enzyme, respectively. One observed strong autoaggregation phenotype, and the isolates demonstrated high activity against *L. innocua*, *L. monocytogenes*, *E. faecalis*, *S. aureus*, *Salmonella* Enteritidis and *Salmonella* Typhimurium. The results instigate the continuity of studies of *E. faecium* isolates in order to obtain a known probiotic strain.

Key words: *Enterococcus*, good bacteria, pathogenic genes, foods, antimicrobial activity.

INTRODUCTION

The use of *Enterococcus* spp. in the making of fermented foods, such as milk, yogurt, cheese, fermented sausages and vegetables (Foulquié Moreno et al., 2006) has a long record in the history of food. Selected *Enterococcus* strains have been employed as probiotics in the promotion of both human and animal health, improving the intestinal microbial balance (Foulquié Moreno et al., 2006; Franz et al., 2011) and producing enterocins (antimicrobial peptides) to inhibit the growth of food-borne pathogenic and spoilage bacteria (Ogaki et al., 2016).

Other therapeutic or prophylactic properties associated with probiotic enterococci include the improvement of constipation and diarrhea, reduction in cholesterol levels, stimulation of immunity and suppression of the carcinogenesis (Agerholm-Larsen et al., 2000; de Roos and Katan, 2000; Parvez et al., 2006; Meurman and Stamatova, 2007; Candela et al., 2008).

However, presence of enterococci in foods may present conflicting effects, either as a risk, a foreign (?) or as an indicator of poor hygiene during the processing of

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food (Bhardwaj et al., 2008). Some types of *Enterococcus* produce virulence factors (Jett et al., 1994; Foulquié Moreno et al., 2006), and are sometimes associated with pathogenicity (Khan et al., 2010). They have been reported to be the cause of endocarditis, bacteraemia, and several infections, besides multiple antibiotic resistances (Kayser, 2003). In addition, vancomycin-resistant *Enterococcus* (VRE) emerged and has become a major public health problem in several countries (Foulquié Moreno et al., 2006).

One cannot presume whether a specific probiotic bacterium will have a beneficial effect on health, except through determination of its genus or species. Reports on the safety of probiotics are limited, and there are few details about the nature of probiotic bacterial species (Sanders et al., 2010; Fijan, 2014). As part of the selection of new probiotic enterococci candidates, one needs to do a series of *in vitro* and *in vivo* analyses to assess their probiotic properties. Carrying no virulence factors nor vancomycin-resistant genes is a prior condition to regard an enterococci candidate as safe and eligible to be used as a starter of cultures, co-cultures; on the other hand, the probiotics that are acceptable for the preparation of food and medicines for humans are those which occur naturally in the intestinal tract of healthy human subjects and foods (Sanders et al., 2010).

Other criteria for potential probiotic strains should include their ability to colonize the intestinal tracts of humans and other mammals (Verschuere et al., 2000), and their resistance to survive humans' biological barriers, such as the strains that have proven ability to survive the gastrointestinal tract (Dunne et al., 2001; Vinderola and Reinheimer, 2003), the presence of proteolytic enzymes and low pH values, bile salts and pancreatic juices.

Probiotic cultures should also be antagonistic to pathogenic bacteria by producing antimicrobial substances and must be safe for human use, maintaining their viability and beneficial properties during manufacturing processes (Schillinger et al., 2005).

Therefore, the objective of this investigation was to perform a characterization of new food enterococcal strains of cheese origin and elicit their potential application as probiotics.

MATERIALS AND METHODS

Bacterial strains and culture preparation

The study comprised one hundred and three *Enterococcus* spp. strains isolated from artisanal soft cheeses. Such isolates were identified as members of the *Enterococcus* spp. based on the phenotypic and genotypic criteria as previously reported (Furlaneto-Maia et al., 2014). A single probiotic culture containing strain *Lactobacillus acidophilus* LA-5 was used as control (Chr. Hansen). The bacterial strains were reactivated in MRS (Merck, Darmstadt, Germany) broth medium for 18 h at 37°C under shaking conditions. Cells were harvested by centrifugation at 10000 g for 5 min and washed twice in NaCl solution (0.85% w/v). The pellet was

resuspended in physiologic solution in order to obtain a suspension that contained approximately 10^9 - 10^{10} CFU/mL.

Antimicrobial susceptibility testing

Antibiotic discs (Laborclin®) were used to determine the strains susceptibility to ampicillin (AMP, 10 µg), nalidixic acid (NAL, 30 µg), vancomycin (VAN, 30 µg), erythromycin (ERY, 15 µg), chloramphenicol (CLO, 30 µg), norfloxacin (NOR, 10 µg), tetracycline (TET, 30 µg), imipenem (IPM 10 µg), amikacin (AK, 30 µg); cephalothin (CF, 30 µg); ciprofloxacin (CIP 5 µg); amoxicillin/clavulanic acid (AMC, 30 µg). The discs were placed onto Mueller–Hinton agar plates overlaid with the enterococcal culture with cell concentration corresponding to 0.5 McFarland standard turbidity. After incubation at 37°C for 18-24 h, the diameter of inhibition haloes around the colonies was measured. Susceptibility or resistance was interpreted in accordance with the Clinical Laboratory Standard Institute (CLSI, 2011) recommendations, and *Staphylococcus aureus* 25923 ATCC were used as strain quality control.

Determination of virulence factors

Enterococcus spp. genomic DNA was extracted by boiling method (Furlaneto-Maia et al., 2014). Determination of virulence factors was performed using a polymerase chain reaction (PCR) method. PCR assay was carried out using species-specific primers (Table 1). All PCR amplifications were performed in a final volume of 20 µl containing 1 pmol of each primer (Forward e Reverse), 0.17 mM dNTPs, 2.5 mM MgCl₂, 1 U of Taq DNA polymerase (Invitrogen), buffer of Taq, and 10 µl template DNA. One observed an initial cycle of denaturation (94°C for 2 min), followed by 30 cycles of denaturation (94°C for 1 min), annealing at an appropriate temperature (Table 1) for 1 min and elongation (72°C for 10 min). A thermal cycler (Techne-Tc3000) was used to perform the PCR reactions. PCR products were analysed by gel electrophoresis in 1.5% agarose stained with ethidium bromide (0.5 g·ml⁻¹), observed under UV transillumination and photographed with L-PIX ST (LOCCUS).

Hemolytic activity

To investigate the production of hemolysin, the strains grown in MRS broth were streaked onto layered agar plates with 7% v/v fresh sheep blood (Himedia), then grown at 37°C for 48 h. β-hemolysis was revealed by the formation of clear zones surrounding the colonies on the blood agar plates (Foulquié Moreno et al., 2006).

Effects of low pH on growth rate

The effects of low pH on growth rate were determined as previously described by Oluwajoba et al. (2013), with modifications. *Enterococcus* spp. bacterial colonies were incubated for 0, 1, 2, 3 and 4 h at 37°C in MRS medium, then adjusted to pH 3 with HCl (4 mol/l). The number of CFU/ml was calculated and compared to the CFU/ml at time 0. The surviving bacteria were counted on the MRS agar, and all these experiments were performed in triplicate.

Lysozyme, bile salts and pancreatin resistance

To simulate the saliva *in vitro*, 200 µL of the bacterial suspensions were inoculated in a sterile electrolyte solution-SES (0.22 g/L

Table 1. Primers used for PCR amplification of virulence genes in *Enterococcus* sp.

Primer	Sequence (5'- 3')	Ta (°C)	bp	Reference
<i>cylA</i>	ACTCGGGGATTGATAGGC GCTGCTAAAGCTGCGCTT	54	688	Creti et al. (2004)
<i>asa1</i>	GCACGCTATTACGAACATATGA TAAGAAAGAACATCACCACGA	56	375	Galli et al. (1990)
<i>gelE</i>	GTTTCATGTCTATTTTCTTCAC CTTCATTATTTACACGTTTG	56	402	Mannu et al. (2003)
<i>ace</i>	AAAGTAGAATTAGATCCACAC TCTATCACATTCGGTTGCC	56	320	
<i>cpd</i>	TGGTGGGTTATTTTCAATTC TACGGCTCTGGCTTACTA	50	782	Eaton and Gasson (2001)

Ta(°C): Annealing temperature; bp: base pairs; *cylA*: cytotoxin; *asa1*: aggregation substance; *gelE*: gelatinase; *ace*: collagen-binding protein; *cpd*: sex pheromone

CaCl₂, 6.2 g/L NaCl, 2.2 g/L KCl, 1.2 g/L NaHCO₃) in the presence of 100 mg/L of lysozyme (Sigma-Aldrich) in accordance with Vizoso-Pinto et al. (2006). Bacterial suspensions in SES without lysozyme were included as control. Samples were incubated at 37°C and microbial counts after 0, 30 and 120 min were carried out on MRS agar (24-48 h; 37°C). Survival rate was calculated as percentage of the CFU/mL after 30 and 120 min in comparison to the CFU/mL at time 0.

Resistance to bile salts and pancreatin was measured as described by Charteris et al. (1998), with modifications. The overnight culture was adjusted to pH 8 and a solution of bile salts (Oxoid) was added to a final concentration of 0.3% or 0.1 g/ml of Pancreatin (Sigma). The mixture (bile salt/ bacterial cells and pancreatin/bacterial cells) was incubated for 0 and 240 min at 37°C. Aliquots were taken for determination of CFU onto the MRS agar. The plates were incubated for 48 h. The addition of bile salt was omitted in the control tube. Results were expressed as percentage of growth as compared to the control (CFU/mL at time zero).

Autoaggregation and co-aggregation assay

The extent of autoaggregation and co-aggregation in the selected probiotic isolates was assessed with the method described by Kos et al. (2003), and the percentage of autoaggregation and co-aggregation was calculated by following Mojgani et al. (2015) descriptions. As to the autoaggregation, overnight-grown cultures of the tested isolates were harvested by centrifugation and the pellet was suspended in PBS (pH 7.0) to obtain an OD (600 nm) of 0.6. The tubes were incubated at 37°C, and the absorbance at 600 nm of the cellular suspensions was monitored every 1 h for a period of 5 h. Co-aggregation assay was performed by mixing equal volumes of a washed-cell suspension of selected probiotic isolates with equal volume of overnight grown cultures of *L. monocytogenes* (CDC 4555). The tubes were incubated at room temperature and absorbance at 600 nm was measured at 5 h. Controls included pure cultures of bacterial cells suspension in PBS.

Screening for enterocin production

The antimicrobial screening assay was evaluated in accordance with Ogaki et al. (2016). Enterococci strains were streaked in plates containing MRS agar, which were then incubated for 24 h at 37°C. The plates were inverted to receive 1 mL of chloroform in the

covers, and remained closed for 20 min. Residual chloroform was evaporated by opening the plates. Using the pour plate method, each indicator strain (10⁸ cells.mL⁻¹) was inoculated into soft MRS agar (0.8%), poured into plates forming an overlay, and these plates were incubated for 24 h at 37°C. If inhibitions zones were found around the colonies, the isolates were considered able to produce enterocin. One used indicator strains such as *Listeria innocua* CLIP 12612, *L. monocytogenes* CDC 4555, *Enterococcus faecalis* ATCC 29212, *S. aureus* ATCC 25925, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538, *Salmonella* Enteritidis ATCC 13076, *Salmonella* Typhimurium UK1 and *Escherichia coli* BAC 49LT ETEC.

Statistical analysis

Statistical analysis was carried out using the software STATISTICA 7 (StatSoft Italia, Padova, Italy). Analysis of variance test (ANOVA) was done in order to determine a significant difference of viability among *Enterococcus* strains and *L. acidophilus*. The collected data were analysed at the significance level of p < 0.05.

RESULTS

Of all strains, 53 were chosen based on their absence of virulence, hemolysis and antimicrobial susceptibility. Almost 2% of the strains showed resistance to vancomycin and eritromycin, and 54% to tetracyclin, while other strains were sensitive to all antimicrobial used.

Twenty-four strains (that is, 45%) were *in-vitro* resistant to bile salt and pancreatic enzyme, ranging from a minimum value of 81.5% to a maximum of 105 and 79.2% to 108.2, respectively (Table 2). The low pH-tolerance property of 24 *Efm* strains was investigated by culturing at pH 3.0 for 120 and 240 min. Of these, seven strains showed higher tolerance, with a survival rate greater than the control strain (*L. acidophilus*) (Table 2), in particular, the *Efm* 55, *Efm* 58, *Efm* 67, *Efm* 9A, *Efm* 16A, *Efm* 19A, *Efm* 44A strains.

Table 2. Tolerance of isolated strains to low pH, bile salts and pancreatic enzymes.

Isolated strain	pH tolerance		Bile salt tolerance	Pancreatic enzyme tolerance
	Survival (%) pH 3.0		Survival (%) 0.3% bile	Survival (%)
	120 min	240 min	240 min	240 min
Efm19	-	-	82.3	99.2
Efm 23	-	-	97.0	83.7
Efm 25	-	-	98.2	87.5
Efm 26	-	-	91.2	95.6
Efm 38	-	-	91.0	85.0
Efm 51	-	-	91.0	79.2
Efm 55	89.3	94.8	82.5	81.1
Efm 58	107.1	99.6	81.5	87.4
Efm 62	-	-	82.0	86.0
Efm 65	-	-	92.0	95.1
Efm 67	91.5	88.2	95.1	96.9
Efm 72	-	-	96.2	83.4
Efm 8A	-	-	83.6	80.6
Efm 9A	93.6	105.2	90.1	87.0
Efm 10A	-	-	89.9	96.7
Efm 11A	-	-	85.5	108.2
Efm 12A	-	-	102.5	83.0
Efm 13A	-	-	98.0	85.7
Efm 15A	-	-	85.2	95.4
Efm 16A	86.0	91.1	105.0	83.3
Efm 19A	98.8	97.0	101.5	90.0
Efm 20A	-	-	87.4	88.9
Efm 26A	-	-	89.5	89.9
Efm 44A	115.8	107.4	92.0	87.8
La	77.8	86.2	81.0	78.6

La: *L. acidophilus*; (-) low survival rate when compared with control bacteria.

In addition, it was studied the survival of these isolates in SES solution containing 100 mg/ml of lysozyme. The isolated strains survived in the presence of lysozyme for 30 and 120 min.

When taken together, results showed that strains Efm 55, Efm 58, Efm 67, Efm 9A, Efm 16A, Efm 19A, Efm 44A were significantly different ($p < 0.05$) in all conditions as compared with the control strain.

According to the autoaggregation results, the Efm9A, Efm19A and Efm67 strains demonstrated strong autoaggregation phenotype, 100, 92 and 50%, respectively, within 5 h of incubation. Moreover, the Efm55, Efm58 strains showed moderate autoaggregation values (45-37%), and the Efm16A did not show any aggregation during the incubation hour. All strains exhibited co-aggregation with strain-pathogen (*L. monocytogenes*), showing values among 65 to 78%.

The antimicrobial spectra of *Enterococcus* strains were investigated by using 9 pathogens as targets. The isolated strains demonstrated broad activity against all tested Gram-positive (*L. innocua* CLIP 12612, *L.*

monocytogenes CDC 4555, *E. faecalis* ATCC 29212, *S. aureus* ATCC 25925, *S. aureus* ATCC 29213, *S. aureus* ATCC 6538) and Gram-negative (*S. Enteritidis* ATCC 13076, *S. Typhimurium* UK1) strains, with halos ranging from 0.4 to 1.52 mm. Although that *E. coli* BAC 49LT ETEC was not inhibited by *Enterococcus* strains.

DISCUSSION

Among the Lactic Acid Bacteria (LAB), members of the *Enterococcus* genus have been object of increasing scientific work, because of its wide range of health-promoting effects. The commonly accepted criteria is that probiotic organisms should be resistant to acid and bile, which are elements present in the stomach and small intestine conditions. In our previous work, the *E. faecium* demonstrated high ability to survive in the presence of lysozyme and pancreatic enzymes, bile salt and low pH, during several hours. More importantly, none of the *E. faecium* strains carried the virulence factors *cylA* and

cytB, required in hemolytic activity, which is the most important virulence trait that lyses the eukaryotic cells (Kayser, 2003). *E. faecium* also showed low antimicrobial resistance, though antimicrobial-resistant probiotics can be used in combination with antimicrobial agents (Sanders et al., 2010).

Based on cell growth /survival, we selected seven *E. faecium* strains for investigation. These strains, initially named as Efm 55, Efm 58, Efm 67, Efm 9A, Efm 16A, Efm 19A, Efm 44A, presented significant activity when compared with the control bacteria.

E. faecium is found in many food products, especially those from animal origin, such as dairy products (Foulquié Moreno et al., 2006; Kivanç et al., 2016). They are most frequently present in many traditional cheeses – prepared mostly from raw ewes' or goats' milk –, and play an important role in the ripening of such products (Manolopoulou et al., 2003). A high prevalence of enterococci in processed foods may be attributed to their resistance to heat, extreme salinity and harsh conditions during the ripening of fermented foods (Gomes et al., 2008; Jurkovic et al., 2006). Altogether, enterococci strains have been a promising probiotic in the promotion of human and animal health by improving the intestinal microbial balance (Foulquié-Moreno et al., 2006; Franz et al., 2011; Buntin et al., 2008).

In this study, Efm strains were exposed to pH 3.0 for 240 min, and several strains were highly resistant to pH 3.0 with levels that were higher than the control bacteria. The average time food stays in the stomach is 3 h, and, in general, our results meet those of other researchers (Mansour et al., 2014).

Once bacteria have survived the gastric barrier (low pH), the environment in the small intestine is a second major barrier for probiotic strains. Therefore, authors have recommended testing bacterial resistance to bile salt concentrations in the 0.3% and pancreatin 0.1 mg/mL to the selection of probiotic bacteria for human use (Bezkorovainy, 2001; Tuomola et al., 2001; Mansour et al., 2014). The major factors determining the survival of LAB include particular characteristics of the strains, tolerance to acid and bile, and resistance to gastric and intestinal juices (Succi et al., 2005). Amaral et al. (2017) and Sun et al. (2010) showed that *E. faecium* was more stable during the simulation of the gastrointestinal tract, showing greater cell viability.

High acidity and high concentration of bile components in the gastrointestinal tract influence the selection of potential probiotic strains (Hyronimus et al., 2000). However, small intestine tolerance is potentially more important than gastric survival. With the development of new delivery systems and the use of specific foods, some studies indicate that acid-sensitive strains can be buffered through the stomach. However, in order to promote a positive effect in the host, probiotics need to survive and colonize his/her small intestine, and the condition of such environment may be an essential

criterion for future probiotics (Huang and Adams, 2004).

This study investigated the antibacterial activity of *E. faecium* strains isolated from soft cheese. These *E. faecium* strains were able to inhibit *L. innocua*, *L. monocytogenes*, *E. faecalis*, *S. aureus*, and *Salmonella*. In particular, *E. coli* was not sensitive to all *E. faecium* strains.

Besides determining that enterococci strains showed high auto-aggregation, one has also demonstrated that they exhibit high co-aggregation against *L. monocytogenes* strain. Aggregation and co-aggregation among bacteria play an important role in the prevention of surface-colonization by pathogens (García-Cayuela et al., 2014), as it is well known that the co-aggregation abilities of LAB strains might interfere with the ability of pathogenic species to infect the host, and can also prevent the colonization of food-borne pathogens (García-Cayuela et al., 2014).

In summary, the results obtained in this study suggest that *E. faecium* strains are resistant to pass through the gastrointestinal tract. One also verified the viability of this strain through the exposure rate and the combination of simulated gastric juice and bile salts, intestinal juice, bile and acid tolerance. Further investigations may be warranted to elucidate its potential health benefit and its application as a promising probiotic strain in the food industry.

Conclusion

This study have demonstrated that *E. faecium* strains of soft-cheese origin may be a probiotic candidate with functional characteristics in terms of resistance to low pH and bile salts, survival under digestion conditions and adhesion, antimicrobial properties, antibiotic resistance, and presence of the virulence factors as well as hemolytic reaction. Further work is in progress to characterize both the bacteriocin(s) and its probiotic functionality.

CONFLICT OF INTERESTS

The authors have not declared any conflict of interests.

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