***Enterococcus faecium* and *Enterococcus durans* isolated from cheese: Survival in the presence of medications under simulated gastrointestinal conditions and adhesion properties**

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ABSTRACT

In this study, we evaluated the survival of *Enterococcus faecium* and *Enterococcus durans*, isolated from cheese, in the presence of medications and under simulated in vitro gastrointestinal conditions. The presence of genes encoding virulence factors, the susceptibility to antimicrobial agents, and adhesion properties were also assessed. *Enterococcus faecium* and *E. durans* both exhibited resistance to most of the tested medications but showed a large sensitivity to analgesics and antihypertensives; they also showed wide susceptibility to antimicrobial agents. *Enterococcus durans* SJRP29 had greater resistance to the presence of medications in comparison with the probiotic *Lactobacillus acidophilus* La-5. The strains, except for *E. durans* SJRP05, did not harbor virulence genes. *Enterococcus durans* SJRP14, SJRP17, and SJRP26 were sensitive to all tested antimicrobial agents. *Enterococcus faecium* was more stable during the simulation of gastrointestinal tract and showed greater viability. At the end of the assay, except for *E. durans* SJRP17, all strains showed high viability ($>7 \log \text{cfu/mL}$). *Enterococcus durans* SJRP29 stood out from the other strains and was selected for further evaluation; it tolerated up to 3.0% NaCl at 30 and 37°C, besides having good adhesion properties (high values of auto-aggregation, co-aggregation, and hydrophobicity). Additionally, the microorganism did not show bile salt hydrolase activity or mucin degradation. These results encourage carrying out additional tests to evaluate the probiotic features by using in vitro dynamic models and in vivo tests before applying these strains to a food system.

Key words: lactic acid bacteria, tolerance to gastrointestinal condition, drug and antibiotic susceptibility, adhesion properties

INTRODUCTION

Enterococcus spp. are lactic acid bacteria (LAB) whose natural habitat is the gastrointestinal (GI) tract of humans and animals. They are also found naturally in vegetables, plant material, and other food. They have specific characteristics, such as the ability to survive in moderately harsh conditions and to maintain viability after heating. Because of this tolerance, *Enterococcus* spp. seem to have a potential application in various food systems. Some evidence suggests that enterococci could have potential applications in the processing of certain fermented dairy products (Giraffa, 2003).

Several studies have evaluated the technological functionality of *Enterococcus faecium*, *Enterococcus faecalis*, and *Enterococcus durans* in combination with mesophilic and thermophilic LAB species (Litopoulou-Tzanetaki et al., 1993; Oumer et al., 2001; Sarantinopoulos et al., 2002). The presence of added enterococcal flora throughout ripening positively affected taste, aroma, color, structure, and the overall sensory profile of different types of cheese. The positive influence of the enterococci on dairy products seems to be due to their lipolytic activity, citrate utilization, and production of aromatic volatile compounds. Enterococci also have the ability to survive to adverse environmental conditions, such as extreme pH, temperatures, and salinity. Consequently, they have been used as components of cheese starter cultures (Giraffa, 2003). Silva et al. (2015) concluded that these microorganisms guarantee the production of a high quantity of lactic acid, which contributes to rapid milk acidification and the production of several flavor and texture compounds.

Additionally, enterococci may produce antibacterial peptides (bacteriocins), generally called enterocins, which can improve products' shelf life and safety (Schirru et al., 2012; Todorov et al., 2011c, 2012, 2014; Favaro et al., 2014). Furthermore, *E. faecium* and *E. faecalis* strains have also been used as probiotics for humans and farm animals (Semedo et al., 2003), and

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the new strains show some synergistic actions with probiotic species, which may result in specific beneficial effects for consumers. However, such a role is still controversial, considering the studies on the determinants of virulence factors that have been performed on *E. faecalis*, *E. faecium*, and *E. durans* (including the food isolates). These studies indicated that these enterococci could be associated with enterococcal nosocomial infections and could harbor multiple antibiotic-resistance genes (Semedo et al., 2003; Favaro et al., 2014).

The beneficial effects of probiotic strains depend on the ability to survive natural host defenses and to multiply in the GI tract (Todorov and Dicks, 2008). The survival of probiotic bacteria under the harsh conditions of the GI tract is a complex process. It depends on the type of diet, availability of nutrients, interaction with GI bacteria, adhesion properties, and auto-aggregation and co-aggregation characteristics (Ahmadova et al., 2013; Todorov et al., 2011c). Therefore, the preliminary evaluation of probiotic microorganisms includes tolerance to GI tract (low pH and presence of bile salts), auto-aggregation, co-aggregation and hydrophobicity properties, presence of the adhesion genes, ability to hydrolyze lactose by β -galactosidase production and safety (absence of antibiotic resistance and virulence factors, and absence of mucolytic activity). After GI tract colonization, the microorganism needs to provide benefits to the host (Jeronymo-Ceneviva et al., 2014).

The survival of *Enterococcus* spp. under simulated GI tract conditions has been already evaluated by several authors, who showed that the strains were able to survive in the GI model, leading to their being considered good candidates for probiotics (Botes et al., 2008; Bhardwaj et al., 2010).

Changes in the diet, stress, or medications commonly used by people (analgesics, antiarrhythmics, antibiotics, antihistamines, proton pump inhibitors, antihypertensives, anti-inflammatories, contraceptives, and other commercial medicaments) may disrupt the microbial balance and cause the number of beneficial strains to decrease. Many individuals who consume probiotics might be simultaneously treating an illness or disorder. Todorov and Dicks (2008), Carvalho et al. (2009), Todorov et al. (2011a), Jeronymo-Ceneviva et al. (2014), and De Paula et al. (2015a) have shown that some medications interfere with the viability of the probiotic by inhibiting their therapeutic potential. As a consequence, a loss of probiotic effectiveness and uncontrolled proliferation of pathogenic bacteria may occur, causing diarrhea and other clinical disorders, such as inflammatory diseases, ulcerative colitis, and cancer (Vinderola et al., 2005; Galdeano et al., 2007).

De Paula et al. (2015a) evaluated the effect of medications in *Leuconostoc mesenteroides* ssp. *mesen-*

teroides SJRP55 and found that among the 65 tested medications, 12 affected the growth of the bacteria. Most of them were analgesic and anti-inflammatory medications containing ibuprofen, butylscopolamine, metamizole, caffeine, orphenadrine citrate, and isometheptene mucate. Another study evaluated the effect of medicaments in LAB isolated from water buffalo mozzarella cheese. All LAB strains were able to survive in the presence of most commercial medications, and the strains were generally inhibited by drugs of the class of anti-inflammatories containing ibuprofen, ketoprofen, and diclofenac potassium (Jeronymo-Ceneviva et al., 2014).

Enterococcus spp. have intrinsic resistance to many antimicrobial agents, such as cephalosporin, β -lactams, and sulfonamides, and low levels of resistance to clindamycin and aminoglycosides. The resistance to chloramphenicol, erythromycin, clindamycin, aminoglycosides, tetracycline, β -lactam (due to the production of β -lactamase or penicillinase), fluoroquinolones, and glycopeptides are usually acquired (Dicks et al., 2009; Tan et al., 2014).

Currently, identifying novel potential probiotic strains, including *Enterococcus* spp., is important because each probiotic strain presents different mechanisms of action and different benefits to the host. Considering that *Enterococcus* spp. can also contribute to the technological and safety characteristics of fermented products, additional research is necessary to identify specific strains and specific characteristics that could also be beneficial for consumers (Rafter, 2004).

This study evaluated the survival of *E. faecium* and *E. durans* isolated from cheese in the presence of medications under simulated in vitro GI conditions, the adhesion properties of the bacteria, and bile salt hydrolase (BSH) activity. The study also determined the susceptibility of the bacteria to antimicrobial agents, the presence of virulence factors, and mucin degradation.

MATERIALS AND METHODS

Isolation and Identification of *Enterococcus* spp.

Ten strains of *E. faecium* and *E. durans* isolated from water buffalo mozzarella cheese samples, collected during storage in 3 independent trials, were used in this study. Based on the DNA gene sequencing, the strains SJRP20, SJRP28, and SJRP65 were previously identified as *E. faecium*, and the others (SJRP05, SJRP14, SJRP17, SJRP25, SJRP26, SJRP29, and SJRP68) were identified as *E. durans* (Silva et al., 2015).

Sterile glycerol (Vetec, Duque de Caxias, RJ, Brazil) was added to the cultures as a cryoprotectant, and cultures were stored in de Man, Rogosa, and Sharpe

(MRS) broth (Difco Laboratories, Detroit, MI) at -80°C . At the time of analyses, the LAB strains were removed from the freezer and thawed at room temperature. To activate the bacteria, the enterococcal strains were inoculated in 10 mL of MRS broth (2%, vol/vol) and incubated at $37^{\circ}\text{C}/24$ h under anaerobic conditions (von Mollendorff et al., 2007), provided by Anaerobac (Probac, São Paulo, SP, Brazil).

Effect of Different Types of Medications on the Viability of Enterococcus Strains

Sixty-two commercial medications, commonly consumed by people and belonging to different groups (analgesic, anti-inflammatory, antihypertensive, and so on), were selected and purchased in a local drugstore. For each medication, a tablet was dissolved in 5 mL of sterile water. The concentration of the substances in the dissolved medication is indicated in Table 1. The sensitivity of enterococcal strains to these medications was evaluated according to Todorov et al. (2011a) and Carvalho et al. (2009). Before the assay, the *Enterococcus* strains were reactivated in MRS broth and incubated at 37°C for 24 h. Then, 100 μL of this culture was spread on the surface of MRS soft agar (0.8%, wt/vol) to achieve a population of 10^6 cfu/mL. The plates were maintained at room temperature for 1 h. After this period, 10 μL of each dissolved medication was added on the surface of MRS and incubated at 37°C for 24 h. The plates with inhibition zones larger than 2 mm in diameter were subjected to the determination of the MIC. The MIC corresponded to the minimal concentration of medication resulting in inhibition halos of at least 2 mm in diameter (Todorov et al., 2011a). The commercial probiotic strain *Lactobacillus acidophilus* La-5 (10^6 cfu/mL, Chr. Hansen, Valinhos, SP, Brazil) was used as a reference strain. The percentage of resistance to the tested medications for each LAB was calculated by the relation between the number of medications to which the strain was resistant and the total number of tested medications. The experiments were performed in duplicate.

Presence of Genes Encoding Virulence Factors, Vancomycin Resistance, and Biogenic Amine Production

The investigation of the presence of genes encoding virulence factors, vancomycin resistance, and biogenic amine production by the *E. faecium* and *E. durans* strains was carried out using the primers and conditions described in Table 2. The amplified products were

separated by electrophoresis on 1% (wt/vol) agarose gels in Tris/borate/EDTA (TBE) buffer. Gels were stained in TBE buffer containing 0.8 $\mu\text{g}/\text{mL}$ ethidium bromide (Sigma-Aldrich, St. Louis, MO).

Susceptibility to Antimicrobial Agents

The susceptibility of 10 enterococcal strains to antimicrobial agents was tested by the agar disk diffusion test using antimicrobial agent disks (Oxoid, São Paulo, SP, Brazil): ampicillin 10 $\mu\text{g}/\text{disk}$, linezolid 30 $\mu\text{g}/\text{disk}$, ciprofloxacin 5 $\mu\text{g}/\text{disk}$, chloramphenicol 30 $\mu\text{g}/\text{disk}$, erythromycin 15 $\mu\text{g}/\text{disk}$, fosfomycin 50 $\mu\text{g}/\text{disk}$, gentamicin 10 $\mu\text{g}/\text{disk}$, nitrofurantoin 300 $\mu\text{g}/\text{disk}$, norfloxacin 10 $\mu\text{g}/\text{disk}$, oxacillin 1 $\mu\text{g}/\text{disk}$, penicillin 10 units/disk, streptomycin 300 $\mu\text{g}/\text{disk}$, teicoplanin 30 μg , tetracycline 30 μg , bacitracin 10 μg , and vancomycin 30 $\mu\text{g}/\text{disk}$.

Reactivation of the strains was performed in MRS broth at 37°C for 24 h, as previously described. A concentration of 10^6 cfu/mL of each enterococcal strain was inoculated in MRS broth supplemented with 0.8% agar. After solidification, disks containing the antimicrobial agents were placed on the MRS agar surface, and incubation was at 37°C for 24 h. The inhibition zones (in mm) around each antimicrobial agents disk was evaluated, and a halo >2 mm was considered positive (Todorov et al., 2011a). The experiments were performed in duplicate.

Survival During the Simulation of GI Tract Conditions

The strains of *E. faecium* and *E. durans* that maintained high viability in the presence of commercial drugs, were susceptible to the tested antimicrobial agents, and showed absence of virulence genes were inoculated into UHT whole milk and evaluated for survival in simulated GI tract conditions. The simulated gastric and enteric conditions were prepared according to Buriti et al. (2010), with modifications. Reactivation of the strains was performed in 10 mL of UHT whole milk at 37°C for 24 h to achieve a population of approximately 10^9 cfu/mL. After the incubation period, 10 mL of UHT whole milk containing the reactivated bacteria was diluted in 90 mL of 0.5% saline solution. This solution was divided into 9 tubes (10 mL in each tube), corresponding to gastric phase, enteric phase I, and enteric phase II, in triplicate.

To simulate the gastric phase, pepsin solution (3 g/L; Sigma-Aldrich) and lipase from *Aspergillus niger* (0.9 mg/L; Sigma-Aldrich) were added to the tubes and the

Table 1. Different types of medications used in the evaluation of *Enterococcus durans* and *Enterococcus faecium* susceptibility

Active substance	Concentration (mg/mL)	Medicine group
Acetylsalicylic acid 20	20	Anti-inflammatory and analgesic
Acetylsalicylic acid 100	100	Anti-inflammatory and analgesic
Betaciclodextrine nimesulide	80	Analgesic with other drugs
Brompheniramine maleate + phenylephrine hydrochloride	2.4 + 3	Decongestant
Carvedilol	1.25	Antianginal and antihypertensive
Celecoxib	40	Anti-inflammatory
Cilostazol	20	Vasodilator
Ciprofibrate	20	Antihyperlipidemia
Clonazepam	0.1	Anticonvulsant and sedative
Clopidogrel bisulfate	15	Antihypertensive
Cloxacolam	0.4	Anxiolytic and sedative
Codeine phosphate + paracetamol	6 + 100	Analgesic
Cyclobenzaprine hydrochloride	1	Muscle relaxant
Cyclobenzaprine hydrochloride	6	Muscle relaxant
Desogestrel	0.015	Contraceptive
Dihydroergotamine mesylate + dipyrone sodium + caffeine	0.2 + 70 + 20	Anti-migraine
Dimenhydrinate + pyridoxine hydrochloride	10 + 2	Antiemetic
Dipyrone sodium ¹	100	Analgesic
Dipyrone sodium ¹	100	Analgesic
Dipyrone sodium + adifenine hydrochloride + promethazine hydrochloride	100 + 2 + 1	Analgesic with other drugs
Dipyrone sodium + isometheptene mucate + caffeine	60 + 60 + 6	Analgesic with other drugs
DL-Methionine + choline chloride + inositol + cyanocobalamin	20 + 5 + 10 + 0.0004	Antihyperlipidemia
Domperidone	2	Antiemetic
Ebastine	2	Antihistamine
Enalapril maleate 2	2	Antihypertensive
Enalapril maleate 4	4	Antihypertensive
Fexofenadine hydrochloride	36	Antiallergic
Flunarizine dihydrochloride	2	Vasodilator
Gestodene + ethinylestradiol	0.015 + 0.06	Contraceptive
Ibuprofen ¹	40	Analgesic and anti-inflammatory
Ibuprofen ¹	40	Analgesic and anti-inflammatory
Kava	46.8	Anxiolytic and sedative
Ketoprofen ¹	10	Anti-inflammatory and analgesic
Ketoprofen ¹	20	Anti-inflammatory and analgesic
Ketoprofen ¹	30	Anti-inflammatory and analgesic
Levothyroxine sodium	0.0176	Action in the thyroid
Loperamide hydrochloride	0.4	Antidiarrheal
Loratadine	2	Antihistamine with other drugs
Losartan potassium	20	Antihypertensive and ACE inhibitor
Meloxicam	3	Anti-inflammatory
Methotrexate sodium	0.5	Antimetabolic
Metoclopramide hydrochloride	2	Antiemetic
Metoprolol succinate	10	Heart failure
Norethindrone acetate + ethinylestradiol	0.4 + 0.02	Antihemorrhagic
Paracetamol ¹	100	Analgesic
Paracetamol ¹	150	Analgesic
Paracetamol ¹	200	Analgesic
Paracetamol + carisoprodol + phenylbutazone	60 + 30 + 15	Analgesic with other drugs
Paracetamol + chlorpheniramine maleate + phenylephrine hydrochloride	80 + 0.8 + 0.8	Action in cold and flu
Paracetamol + pseudoephedrine hydrochloride	100 + 6	Analgesic with other drugs
<i>Passiflora alata</i> + <i>Erythrina mulungu</i> + <i>Crataegus oxyacantha</i>	5 + 2.5 + 2.5	Neuro-sedative
Phloroglucinol	16	Anti-inflammatory
Prednisolone	1	Glucocorticoid
Prednisone	4	Anti-inflammatory
Promethazine hydrochloride	5	Antihistamine
Propranolol hydrochloride	8	Antihypertensive
Rupatadine fumarate	2.56	Antiallergic
Scopolamine butylbromide + dipyrone sodium	2 + 50	Antispasmodic and analgesic
Simvastatine	4	Antilipidemic
Spironolactone	5	Diuretic
Tranexamic acid	50	Antihemorrhagic
Trometamol	2	Analgesic
Valsartan + amlodipine	32 + 1	Antihypertensive

¹Medications with the same name came from different commercial brands.

Table 2. Primer sequences used in the investigation of presence of genes encoding virulence factors, vancomycin resistance, and biogenic amine production (Mannu et al., 2003; Vankerckhoven et al., 2004; de Las Rivas et al., 2005; Martín-Platero et al., 2009)

Target	Gene ¹	Primer	Annealing temperature (°C)	Amplicon size (bp)
Virulence	<i>gelE</i>	GEL 1: AGTTCATGTCTATTTTCTTCAC GEL 2: CTTTCATTATTTACACGTTTG	56	402
	<i>hyl</i>	HYL n1: ACAGAAGAGCTGCAGGAAATG HYL n2: GACTGACGTCCAAGTTTCCA	53	276
	<i>asa1</i>	ASA 11: GCACGCTATTACGAACATGA ASA 12: TAAGAAAGAACATCACCACGA	50	375
	<i>esp</i>	ESP 14F: AGATTTCATCTTTGATTCTTG ESP 12R: AATTGATTCTTTAGCATCTGG	47	510
	<i>cylA</i>	CYT I: ACTCGGGGATTGATAGGC CYT IIb: GCTGCTAAAGCTGCGCTT	52	688
	<i>efaA</i>	EFA-AF: GCCAATTGGGACAGACCCTC EFA-AR: CGCCTTCTGTTCTTCTTTGGC	57	688
	<i>ace</i>	ACE-F: GAATTGAGCAAAAAGTTCGAATCG ACE-R: GTCTGTCTTTTCACTTGTTC	48	1,008
	Antibiotic resistance	VAN-AF: TCTGCAATAGAGATAGCCGC VAN-AR: GGAGTAGCTATCCCAGCATT	52	377
		VAN-BF: GCTCCGCAGCCTGCATGACA VAN-BR: ACGATGCCGCCATCCTCCTGC	60	529
		JV16HC: AGATGGTATTGTTTCTTATG JV17HC: AGACCATACACCATAACCTT	46	367
Biogenic amines	<i>hdc1</i>	106: AAYTCNTTYGAYTTYGARAARGARG 107: ATNGGNGANCCDATCATYTTTRTGNC	50	534
	<i>tdc</i>	P1: CCRTARTCNGGNATAGCRAARTCNGTRTG P2: GAYATNATNGGNATNGGNYTNGAYCARG	55	924
	<i>odc</i>	3: GTNTTYAAYGCNGAYAARACNTAYTTYGT 16: TACRCARAATACTCCNGNGGRTANGG	54	1,446

¹*gelE* = gelatinase; *hyl* = hyaluronidase; *asa1* = aggregation substance; *esp* = enterococcal surface protein; *cylA* = cytolysin; *efaA* = endocarditis antigen; *ace* = adhesion of collagen; *vanA* and *vanB* = vancomycin resistance; *hdc1* and *hdc2* = histidine decarboxylase; *tdc* = tyrosine decarboxylase; *odc* = ornithine decarboxylase.

pH was adjusted to 2.0 to 2.5 with 0.5 N HCl. To simulate the enteric phase I, the pH values of the samples were adjusted from 4.3 to 5.2 with the addition of alkaline solution containing pancreatin (1 g/L; Sigma-Aldrich) and bile salts (10 g/L; Sigma-Aldrich). To simulate the enteric phase II, the pH values of the samples were adjusted from 6.7 to 7.5 with the addition of the same solution, and the concentrations of pancreatin (1 g/L; Sigma-Aldrich) and bile salts (10 g/L; Sigma-Aldrich) were adjusted. The samples were incubated for 120 min at 37°C under agitation of 150 rpm between each step of the assay. After 30, 120, 240, and 360 min of the beginning of the assay, 1-mL aliquots were collected from triplicate samples for the enumeration of viable cells. The serial dilutions were carried out using 0.1% (wt/vol) peptone water, and the bacteria were counted in MRS agar (Difco) applying the pour plate technique. The petri dishes were incubated at 37°C for 48 h in anaerobiosis provided by Anaerobac (Probac).

Selection of Strains for Complementary Tests

Strains that were simultaneously viable in the presence of medications, lacked antibiotic resistance and

virulence genes, and showed high viability (>7 log cfu/mL) under the simulated GI conditions were selected for complementary tests of tolerance to different NaCl concentrations, BSH activity, auto-aggregation and co-aggregation, cell surface hydrophobicity, and absence of mucin degradation, which are essential for probiotic strains.

Tolerance to NaCl

The cells' capacity to tolerate different NaCl concentrations was tested in vitro, according to Todorov et al. (2008). The selected strain was activated in MRS broth at 37°C for 18 h and cultivated in MRS broth adjusted to different NaCl concentrations [0, 0.5, 1.0, 3.0, 5.0, and 7.0 (wt/vol); Synth, Diadema, SP, Brazil]. All tests were conducted in sterile flat-bottom 96-well micro titer plates (TPP, Trasadingen, Switzerland). Each well was filled with 180 µL of modified MRS broth with NaCl and 20 µL of culture. The optical density (OD) was measured at 600 nm (OD_{600 nm} = 0.2). The strain was incubated at 30 and 37°C. The OD_{600 nm} was recorded every hour for 12 h. Experiments were performed in 3 different trials, in triplicate (n = 9).

BSH Activity

The BSH activity was evaluated according to Kumar et al. (2013), with some modifications. Briefly, the selected strain was activated in MRS broth at 37°C for 18 h, and 10 µL of the culture was spotted in MRS agar plates, supplemented with 0.5% (wt/vol) of sodium salt of taurodeoxycholic acid (**TDCA**; T0875, Sigma Aldrich) or taurocholic acid (**TC**; T4009, Sigma Aldrich), and 0.37 g/L of CaCl₂ (Vetec, Duque de Caxias, RJ, Brazil). Plates were incubated anaerobically at 37°C for 72 h. The presence of white precipitated bile acid around spots was considered as a positive result. MRS plates without TDCA or TC were used as a negative control. Experiments were performed in 3 different trials, in triplicate (n = 9).

Auto-Aggregation, Co-Aggregation, and Hydrophobicity Assays

The selected strain was activated in MRS broth at 37°C for 24 h and harvested by centrifugation (7,000 × g at 20°C for 10 min), washed twice, and diluted in sterile saline solution 0.85%. One milliliter of cell suspension was transferred to a 2-mL sterile Eppendorf tube to obtain the OD at 660 nm (OD_{660 nm} = 0.3), and the samples were incubated at 4, 37, and 42°C. After 60 min, the cell suspension was centrifuged (300 × g, 20°C for 2 min) and the OD_{660 nm} of the supernatant was determined. The auto-aggregation was determined using Equation [1] (Todorov et al., 2011a), wherein OD₀ refers to initial OD and OD₆₀ refers to the OD value measured after 60 min:

$$\text{Auto-aggregation (\%)} = \left[(\text{OD}_0 - \text{OD}_{60}) / \text{OD}_0 \right] \times 100. \quad [1]$$

For the co-aggregation assay, the strains and the indicating microorganisms *L. fermentum* (SJRP30) and *L. casei* (SJRP37) were cultivated in 10 mL of MRS broth and the strains of *Listeria monocytogenes* ATCC 7644 and *Listeria innocua* ATCC 33090 in brain heart infusion (**BHI**, Difco) at 30°C. The cell preparation and the experimental protocol for the study of co-aggregation was the same used for auto-aggregation. The co-aggregation trials were performed in presence of 500 µL of selected LAB cells in combination with 500 µL of the indicating microorganism cells in a sterile plastic cuvette. Co-aggregation was determined using Equation [2] (Todorov et al., 2011a), wherein OD₀ refers to initial OD and OD₆₀ refers to the OD value measured after 60 min. All experiments were performed in 3 different trials, in triplicate (n = 9):

$$\text{Co-aggregation (\%)} = \left[(\text{OD}_0 - \text{OD}_{60}) / \text{OD}_0 \right] \times 100. \quad [2]$$

The ability of the selected LAB cells surface to adhere to hydrophobic compounds was evaluated according to the methods reported by Todorov and Dicks (2008) and Doyle and Rosenberg (1995). The cells were centrifuged (6,700 × g at 4°C for 6 min), washed twice in phosphate buffer 0.1 M, suspended in the same solution, and the OD_{580 nm} was measured (OD₀). Cell suspension (1.5 mL) was added to 1.5 mL of n-hexadecane (Sigma Aldrich) and vortexed for 2 min. The aqueous and organic phases were separated at room temperature for 30 min. An aliquot of 1 mL of the aqueous phase was removed to determine the OD_{580 nm}. The percentage of hydrophobicity was determined by Equation [3], wherein OD₀ refers to the initial OD value and the OD₃₀ refers to OD value measured after 30 min. The experiments were performed in 3 different trials, in triplicate (n = 9):

$$\text{Hydrophobicity (\%)} = \left[(\text{OD}_0 - \text{OD}_{30}) / \text{OD}_0 \right] \times 100. \quad [3]$$

Mucin Degradation

The selected LAB strain's capacity to degrade mucin was evaluated according to Peres et al. (2014) and Zhou et al. (2001). The cultures were activated in MRS broth and incubated at 37°C for 18 to 24 h. Agar plates were prepared using the basal medium described by Zhou et al. (2001). Then, 10 µL of the selected LAB strain was inoculated by spotting onto the surface of the agar plates, which were incubated at 37°C for 48 h anaerobically. After the incubation, plates were stained for 30 min with 0.1% amide black (wt/vol) (Merck Millipore Ltd., Cork, Ireland) prepared in 3.5 M acetic acid and washed with 1.2 M acetic acid. Mucin lyse zones were observed as discolored halos that indicated mucin degradation activity around the colonies. *Escherichia coli* ATCC 8739 and *Salmonella enterica* ssp. *enterica* serovar Typhimurium ATCC 14028 were used as a positive control and *Lactobacillus rhamnosus* GG (ATCC 53103) as a negative control. The experiments were performed in 2 different trials, in triplicate (n = 6).

Statistical Analysis

The results of viability in the presence of drugs, susceptibility to antibiotics, tolerance to NaCl, aggregation, co-aggregation, and hydrophobicity were analyzed through the average calculation and standard deviation. The results of survival during the simulation of GI tract conditions were examined by ANOVA in a completely randomized design in 3 replicates, using the software ESTAT version 2.0 (UNESP, Jaboticabal, SP, Brazil). Mean values were compared using the Tukey's test at 5% significance level.

RESULTS AND DISCUSSION

Viability in the Presence of Medications

Some people who consume probiotic foods may be simultaneously receiving medical treatments. Therefore, determining the effect of medications on the survival of potential probiotic strains is important. The 10 strains of *E. faecium* and *E. durans* were resistant to most of the medications (Table 3). Some medications had a strong effect on the *Enterococcus* strains, including analgesics (containing ibuprofen, paracetamol, and metamizole, which are available without medical prescription and commonly used by people of different ages, from babies to elderly people) and antihypertensive (containing valsartan, amlodipine, and enalapril maleate, often administered in continuous use for patients with coronary heart disease), anti-inflammatory, and antidiarrheal medications. In contrast, the antihypertensive and antidiarrheal drugs had a smaller effect on the growth of *L. acidophilus* La-5 (Table 3).

Almost all enterococcal strains and the probiotic strain of *L. acidophilus* La-5 were inhibited by ibuprofen (40 mg) and paracetamol (150 mg/mL). The strain *E. durans* SJRP29 was not inhibited by ibuprofen (40 mg/mL), and *E. durans* SJRP68 and *E. faecium* SJRP28 were not affected in the presence of paracetamol (Table 3). The interference of medications containing ibuprofen and diclofenac on the survival of probiotics may be a consequence of an increased concentration of potassium ions in the gastric contents because of the dissolution of diclofenac potassium in the stomach. The excess of potassium ions in the medium affects the viability of the bacterial cells (Carvalho et al., 2009; Todorov et al., 2011b).

None of the enterococcal strains had their growth affected by neuro-sedative (composed of *Passiflora alata*, *Erythrina mulungu*, and *Crataegus oxyacantha*), antimetabolic (methotrexate), antimigraine (composed of dihydroergotamine mesylate, dipyron sodium, and caffeine), antiemetic (domperidone), and analgesics (dipyron sodium and ketoprofen); however, growth of the probiotic strain *L. acidophilus* La-5 was inhibited by these medications. Additionally, the tolerance of enterococcal strains to methotrexate (2,4-diamino-N10-methyl propylglutamic acid) was quite high, whereas *L. acidophilus* La-5 was inhibited by low MIC (<0.01 mg); that is, it was able to inhibit the strain even at a very low concentration. The resistance to methotrexate is important because it is applied in different treatments (solid tumors, hematologic malignancies, multiple sclerosis, Crohn's disease, and rheumatoid arthritis), and enterococcal strains could be recommended for reducing the side effects of this medication on the GI microbiota.

Enterococcus durans SJRP05, SJRP17, SJRP29, and SJRP68 strains were less inhibited in the presence of medications (Table 3). *Enterococcus durans* SJRP29 showed optimal survival in the presence of medications (resistant to 85.48% of the tested medications). Despite being sensitive to paracetamol, it was resistant to other analgesics, such as ibuprofen and analgesic containing dipyron sodium, adifenine hydrochloride, and promethazine hydrochloride, whereas the probiotic strain *L. acidophilus* La-5 was sensitive to all of them. Additionally, *E. durans* SJRP29 also presented notably high values for the MIC test compared with the other strains, which means that this strain was not inhibited by the tested medications even at high concentrations; the probiotic strain *L. acidophilus* La-5 was one of the most affected by the medications (sensitive to 27.4% of the tested medications, Table 3).

Because the medications may interfere with the viability of enterococci and thereby reduce the therapeutic effects of their probiotic properties, strains that maintain their viability for potential application in food or supplements must be selected. In addition, people who consume medications and probiotics simultaneously must be notified of this possibility.

Several studies have shown evidences of these medications' interferences in the probiotic properties and benefits of *L. acidophilus* (Todorov et al., 2011b); *Enterococcus mundtii* and *Lactobacillus plantarum* (Botes et al., 2008); *Lactobacillus casei* SJRP35, *Leuconostoc citreum* SJRP44, *Lactobacillus delbrueckii* ssp. *bulgaricus* SJRP57, and *Leuconostoc mesenteroides* ssp. *mesenteroides* SJRP58 (Jeronymo-Ceneviva et al., 2014); and *Leuconostoc mesenteroides* ssp. *mesenteroides* SJRP55 (De Paula et al., 2015a). In these studies, the effects of anti-inflammatories, diuretics, neuroleptics, analgesics, antipyretics, antifungals, contraceptives, and others were evaluated.

The natural intestinal bacteria may also be affected by the use of medications. The reduction of the number of beneficial bacteria naturally present in the intestine could result in an imbalance of the GI microbiota. This effect is based on the absorption of the medications through the GI tract, which depends on the breakdown by the enzymes present in the gut and released by the microbiota and on the interaction with food and beverages, which may decrease or increase the absorption. Considering this drug metabolism, further studies should be conducted to better understand the interactions between intestinal strains and medications. Moreover, physicians and pharmacists can better recommend which probiotic should be used simultaneously with medications in a specific case to prevent failure of the therapeutic effect of probiotics.

Table 3. Effect of commercial medications on the survival of *Enterococcus durans* and *Enterococcus faecium*, presented as diameter¹ of inhibition zones in millimeters and minimal inhibitory concentration (MIC)

Active substance	<i>Enterococcus durans</i>							<i>Enterococcus faecium</i>			<i>Lactobacillus acidophilus</i>
	SJRP05	SJRP14	SJRP17	SJRP25	SJRP26	SJRP29	SJRP68	SJRP20	SJRP28	SJRP65	La-5
Acetylsalicylic acid 20	R	R	R	R	R	R	R	R	R	R	R
Acetylsalicylic acid 100	R	R	R	R	R	R	R	R	R	R	R
Betacloxdexrine nimesulide	R	R	R	R	R	R	R	R	R	R	R
Brompheniramine maleate + phenylephrine hydrochloride	R	R	R	R	R	R	R	R	R	R	R
Carvedilol	R	R	R	R	R	R	R	R	R	R	R
Celecoxib	R	R	R	R	R	R	R	R	R	R	R
Cilostazol	R	R	R	R	R	R	R	R	R	R	R
Ciprofibrate	14.5 (5)	20 (10)	20.5 (20)	14.5 (5)	20.5 (20)	R	16.5 (5)	16 (5)	21.5 (5)	16.5 (5)	11.0 (20)
Clonazepam	R	R	R	R	R	R	R	R	R	R	R
Clopidogrel bisulfate	R	R	R	R	R	R	R	R	R	R	R
Cloxacolam	R	R	R	R	R	R	R	R	R	20.0 (0.4)	R
Codeine phosphate + paracetamol	R	R	R	15.0 (106)	R	R	R	R	12.5.0 (106)	R	R
Cyclobenzaprine hydrochloride	R	R	R	R	R	R	R	R	R	R	R
Desogestrel	R	R	R	R	R	R	R	R	R	R	R
Dihydroergotamine mesylate + dipyrone sodium + caffeine	R	R	R	R	R	R	R	R	R	R	20 (0.1; 10; 35)
Dimenhydrinate + pyridoxine hydrochloride	R	R	R	R	R	R	R	R	R	R	R
Dipyrone sodium	22.5 (12.5)	22.5 (25)	20.0 (50)	19.0 (25)	17.5 (12.5)	R	21.5 (25)	17.0 (12.5)	R	18.5 (12.5)	15.0 (100)
Dipyrone sodium	R	R	R	R	R	R	R	R	R	R	22.5 (25)
Dipyrone sodium + adifenine hydrochloride + promethazine hydrochloride	11 (50; 1.0; 0.5)	12.5 (100; 2; 2)	R	17 (100; 2; 1)	12.0 (100; 2; 1)	R	15.5 (100; 2; 1)	R	12.0 (100; 2; 1)	21.0 (50; 1.0; 0.5)	17.5 (25; 0.5; 0.25)
Dipyrone sodium + isometheptene mucate + caffeine	R	R	R	R	R	R	R	R	R	R	13.0 (30; 3; 3)
Di-Methionine + choline chloride + inositol + cyanocobalamin	R	R	R	R	R	R	R	R	R	R	R
Domperidone	R	R	R	R	R	R	R	R	R	R	30.0 (0.125)
Ebastine	R	R	R	R	R	R	R	R	R	R	R
Enalapril maleate 2	R	R	R	R	R	R	R	14.0 (2)	R	R	23.5 (2)
Enalapril maleate 4	R	32.5 (0.0625)	48.0 (0.0625)	34.5 (0.0625)	31.0 (0.0625)	29.5 (0.0625)	32.0 (0.0625)	34.0 (0.0625)	37.0 (0.0625)	36.0 (0.0625)	R
Fexofenadine hydrochloride	R	R	R	R	R	18.5 (36)	R	15.5 (18)	R	R	R

Continued

Table 3 (Continued). Effect of commercial medications on the survival of *Enterococcus durans* and *Enterococcus faecium*, presented as diameter¹ of inhibition zones in millimeters and minimal inhibitory concentration (MIC)

Active substance	<i>Enterococcus durans</i>								<i>Enterococcus faecium</i>				<i>Lactobacillus acidophilus</i>
	SJRP05	SJRP14	SJRP17	SJRP25	SJRP26	SJRP29	SJRP68		SJRP20	SJRP28	SJRP65		La-5
Flunarizine dihydrochloride	R	R	R	R	R	R	R		R	R	R		R
Gestodene + ethinylestradiol	R								R				R
Ibuprofen ²	18.5 (10)	16.5 (10)	R	14.0 (20)	19.0 (20)	20.5 (0.75)	13.5 (20)		14.5 (20)	23.0 (0.019)	16.5 (20)		20.0 (20)
Ibuprofen ²	17.5 (5)	11.5 (20)	14.0 (40)	16.0 (40)	1.0 (20)	R	15.5 (20)		13.5 (20)	19.5 (20)	20.0 (20)		20.0 (20)
Kava	21.5 (23.4)	16.5 (46.8)	R	R	14.5 (46.8)	20.5 (46.8)	16.5 (46.8)		15.0 (46.8)	19 (46.8)	23.0 (23.4)		R
Ketoprofen ²	15.5 (0.625)	17.5 (1.25)	18.0 (2.5)	13.0 (2.5)	20.0 (1.25)	R	15.5 (2.5)		20.5 (0.156)	19.0 (1.25)	21.5 (1.25)		R
Ketoprofen ²	R	R	R	R	R	R	R		R	R	R		13.0 (20)
Ketoprofen ²	R	R	R	R	R	R	R		R	R	R		15.0 (30)
Levothyroxine sodium	20 (0.004)	16.5 (0.004)	14.5 (0.0176)	15.0 (0.004)	17.5 (0.008)	R	15.0 (0.0176)		17.0 (0.004)	R	24.5 (0.004)		R
Loperamide hydrochloride	16.0 (0.2)	16.0 (0.2)	17.5 (0.2)	19.0 (0.4)	18.0 (0.4)	17.0 (0.4)	13.0 (0.4)		16.5 (0.4)	18.5 (0.4)	16.0 (0.2)		R
Loratadine	R	6.0 (2)	R	18.0 (2)	R	R	R		R	R	R		27.5 (0.125)
Losartan potassium	R	10 (20)	R	R	R	R	R		R	R	R		R
Meloxicam	R	17.5 (3)	R	R	R	R	R		R	R	R		R
Methotrexate sodium	R	R	R	R	R	R	R		R	R	R		35.0 (<0.01)
Metoclopramide hydrochloride	R	R	R	R	R	R	R		R	R	R		R
Metoprolol succinate	R	16.0 (10)	15.0 (10)	16.0 (10)	19.5 (10)	15.0 (10)	R		12.0 (5)	16.0 (10)	R		R
Norethindrone acetate + ethinylestradiol	R	R	R	R	R	R	R		R	R	R		R
Paracetamol + carisoprodol + phenylbutazone	R	R	R	R	R	R	R		R	R	R		R
Paracetamol + chlorpheniramine maleate + phenylephrine hydrochloride	R	R	R	R	R	R	R		R	R	R		R
Paracetamol + pseudoephedrine hydrochloride	R	R	R	R	R	R	R		R	R	R		R
Paracetamol ²	22.5.0 (75)	16.5 (150)	12.5 (75)	12.5 (150)	15.0 (150)	15.5 (150)	R		13.0 (150)	R	14.0 (150)		16.0 (75)
Paracetamol ²	R	14.5 (200)	R	R	15.5 (200)	R	R		12.5 (100)	R	17.0 (200)		R

Continued

Table 3 (Continued). Effect of commercial medications on the survival of *Enterococcus durans* and *Enterococcus faecium*, presented as diameter¹ of inhibition zones in millimeters and minimal inhibitory concentration (MIC)

Active substance	<i>Enterococcus durans</i>							<i>Enterococcus faecium</i>			<i>Lactobacillus acidophilus</i>
	SJRP05	SJRP14	SJRP17	SJRP25	SJRP26	SJRP29	SJRP68	SJRP20	SJRP28	SJRP65	La-5
<i>Passiflora alata</i> + <i>Erythrina mulungu</i> + <i>Crataegus oxyacantha</i>	R	R	R	R	R	R	R	R	R	R	16.0 (2; 1.25; 1.25)
Phloroglucinol	R	R	R	R	R	R	R	R	R	R	R
Prednisolone	R	R	R	R	R	R	R	R	R	R	R
Promethazine hydrochloride	R	R	R	R	R	R	R	R	17.0 (2.5)	R	R
Propranolol hydrochloride	R	R	R	R	R	R	R	R	R	R	R
Rupatadine fumarate	R	R	17.0 (2.56)	R	R	R	R	R	R	R	R
Scopolamine butylbromide + dipyrone sodium	R	R	R	R	R	R	R	R	R	R	15.0 (1.25)
Simvastatine	13.0 (1)	R	R	R	R	R	R	R	R	R	R
Spironolactone	R	13.0 (5)	R	R	15.5 (5)	R	R	R	14.5 (5)	R	R
Tranexamic acid	R	R	16.5 (50)	R	R	R	R	R	22.0 (50)	R	R
Trometamol	R	R	R	R	R	R	R	R	R	R	R
Valsartan + amlodipine	15.5 (1.03)	22.0 (2.06)	22.0 (2.06)	18.0 (2.06)	27.0 (2.06)	23.5 (8.25)	16.5 (4.125)	22.0 (1.03)	25.0 (0.51)	23.0 (1.03)	R
Resistance to the tested medications (%)	80.65	70.97	80.65	77.42	75.81	85.48	82.26	77.42	74.19	77.42	72.58

¹R = resistant.

²Medications with the same name are from different manufacturers.

Table 4. Effect of antibiotics on *Enterococcus durans* and *Enterococcus faecium*¹

Antibiotic ²	<i>Enterococcus durans</i>							<i>Enterococcus faecium</i>			<i>Lactobacillus acidophilus</i> La-5
	SJRP05	SJRP14	SJRP17	SJRP25	SJRP26	SJRP29	SJRP68	SJRP20	SJRP28	SJRP65	
S 300	S	S	S	S	S	S	S	S	S	S	S
CN 10	S	S	S	S	S	S	S	S	S	S	S
P 10	S	S	S	S	S	S	S	S	S	S	S
B 10	S	S	S	S	S	S	S	S	S	S	S
TE 30	S	S	S	S	S	S	S	S	S	S	S
C 30	S	S	S	S	S	S	S	S	S	S	S
VA 30	S	S	S	S	S	S	S	S	R	S	S
AMP 10	S	S	S	S	S	S	S	S	S	S	S
LZD 30	S	S	S	S	S	S	S	S	S	S	S
FOS 50	S	S	S	S	S	S	S	S	R	S	R
CIP 5	S	S	S	S	S	S	S	S	S	S	S
E 15	S	S	S	S	S	S	S	S	S	S	S
NOR 10	S	S	S	S	S	S	S	S	S	S	S
F 300	S	S	S	S	S	S	S	S	S	S	S
OX 1	R	S	S	R	S	R	R	R	S	R	S
TEC 30	S	S	S	S	S	S	S	S	R	S	S

¹Strains with inhibition halos >2 mm were considered sensitive (S), and strains with halos <2 mm were considered resistant (R).

²AMP 10 = ampicillin 10 µg; LZD 30 = linezolid 30 µg; CIP 5 = ciprofloxacin 5 µg; C 30 = chloramphenicol 30 µg/disk; E 15 = erythromycin 15 µg/disk; FOS 50 = fosfomycin 50 µg/disk; CN 10 = gentamicin 10 µg/disk; F 300 = nitrofurantoin 300 µg/disk; NOR 10 = norfloxacin 10 µg/disk; OX 1 = oxacillin 1 µg; P 10 = penicillin 10 units/disk; S 300 = streptomycin 300 µg/disk; TEC 30 = teicoplanin 30 µg; TE 30 = tetracycline 30 µg/disk; B 10 = bacitracin 10 µg; VA 30 = vancomycin 30 µg/disk.

Sensitivity to Antimicrobial Agents

Most of the enterococcal strains were sensitive to the majority of antimicrobial agents; however, resistance to the antibiotics oxacillin, teicoplanin, fosfomycin, and vancomycin was observed (Table 4). The strains *E. durans* SJRP05, SJRP25, SJRP29, and SJRP68 and *E. faecium* SJRP20 and SJRP65 were only resistant to oxacillin. The reference probiotic strain *L. acidophilus* La-5 showed resistance toward fosfomycin. In contrast, the guidance on the assessment of bacterial susceptibility to antimicrobials of human and veterinary importance, proposed by the European Food Safety Authority (2012) for gram-positive bacteria, does not include oxacillin, teicoplanin, and fosfomycin, probably because this kind of resistance is considered an intrinsic feature of LAB and chromosomally encoded.

Resistance to antimicrobial agents among different LAB isolated from fermented food (yogurt, fermented sausages, cheese) and commercial probiotic bacteria has already been documented (Lee et al., 2008; Drago et al., 2013; Morandi et al., 2013).

Werner et al. (2013) reported that enterococci are often resistant to antibiotics such as erythromycin, tetracycline, kanamycin, penicillin, and vancomycin. Drago et al. (2013) observed specific antibiotic resistance traits in many Italian and European probiotic strains. These traits may be a consequence of the acquisition of mobile genetic elements that encode antimicrobial resistance, leading to the spread of a variety of antimicrobial resistance determinants. *Enterococcus* strains

isolated from retail shrimp presented high resistance to antimicrobial agents; more than half of all investigated isolates (65.7%) were resistant to at least one class of antibiotics, and 16 (45.7%) were classified as multidrug resistant. A high percentage of isolates were resistant to tetracycline (48.6%), followed by tigecycline and fosfomycin (45.7%; Chajęcka-Wierzchowska et al., 2016).

The concern about resistance to antimicrobial agents is partly due to the indiscriminate use of this kind of medicine by human beings, agriculture, and livestock. Pathogenic bacteria, LAB, and opportunistic bacteria can then acquire or transmit resistance to antimicrobial agents and virulence genes via transposons and plasmids spread by conjugation or transformation (Gratetpanche et al., 2008).

Enterococcus durans SJRP14, SJRP17, and SJRP26 strains were sensitive to all antimicrobial agents tested, including oxacillin, to which most strains were resistant. The other enterococcal strains showed similar results regarding susceptibility to antimicrobial agents. Morandi et al. (2013) also observed that none of the studied strains of *Enterococcus lactis* were classified as resistant to clinically important antibiotics such as ampicillin, erythromycin, penicillin G, tetracycline, and vancomycin. Furthermore, PCR-based detection did not identify any of the common genetic determinants for vancomycin, tetracycline, and erythromycin resistance.

Over the last years, enterococci have demonstrated a potential to harbor and transfer resistance genes. Courvalin (2006), Ogier and Serror (2008), Hollenbeck

and Rice (2012), and more recently Sharma et al. (2014) published extensive and valuable reviews that summarize the multiple aspects of *Enterococcus* spp.: their role in dairy products (especially in cheese), the safety of commercial probiotic bacteria, the effect of opportunistic pathogens on human health, antibiotic multiresistance, the biochemistry of resistance, the genetics of resistance, and the potential for spreading resistance genes and virulence factors. These factors illustrate the controversial nature of this bacterial genus. Acquired resistance can be due to 2 distinct mechanisms: (1) mutations in endogenous (housekeeping) genes, in which case resistance is not transferable, and (2) acquisition of an exogenous resistance determinant from another bacterium by horizontal (lateral) gene transfer, in which case the gene can be transferable (Courvalin, 2006). The safety concern for such microorganisms arises from the possibility of the resistance spreading to harmful microorganisms or commensal gut microbiota. This transfer can be a major concern if the gene is carried by a mobile genetic element, especially a self-transferable plasmid or transposon. The resistance of *E. faecium* and *E. faecalis* to macrolides, tetracyclines, chloramphenicol, fosfomycin, rifampin, and quinolones is very common, and the resistance to glycopeptides, such as vancomycin and teicoplanin, is due to the synthesis of an alternative cell wall and depends on the phenotype. The mobile genetic elements (transposon Tn1546 and Inc18 plasmids) are responsible for most gene mobility in enterococci and confer glycopeptide resistance (Hollenbeck and Rice, 2012).

Considering that enterococci can be used for a starter culture (starter) in many dairy products, especially because they produce particular sensory characteristics for the food products (Moraes et al., 2012), the concern is that enterococci could contribute to the horizontal spread of virulence factors to pathogenic or commensal bacteria present in the GI tract (De Paula et al., 2015b; Tejedor Junco et al., 2015). The microbial population found in the human intestine represents a huge reservoir of antimicrobial resistance genes, whose ingestion could influence the presence, establishment, and dynamics of resistance of bacteria to antimicrobial agents in the body (Gueimond et al., 2013). Therefore, the use of enterococci in foods, such as commercial starters and probiotic strains, should be possible on a case-by-case basis once studies establish their harmlessness or at least the lack of acquired antibiotic resistance genes and proven virulence factors (Ogier and Serror, 2008; Grattepanche et al., 2008; Favaro et al., 2015). Additionally, efforts must continue to prevent the development of antibiotic resistance and spread in enterococci (Hollenbeck and Rice, 2012).

Virulence Factors

Factors that may enhance the virulence of bacteria include antibiotic resistance determinants, cytolytic toxin, gelatinase, aggregation substance, extracellular superoxide production, and enterococcal surface protein (Tejedor Junco et al., 2015). The need for an effective risk assessment of new enterococcal strains is also strengthened by the concern for their presence and especially their use in food products (Semedo et al., 2003). In our study, except for *E. durans* SJRP05, the strains did not harbor virulence genes, such as *gelE*, *cylA*, *hyl*, *asa1*, *esp*, *efaA*, *ace*, *vanA*, *vanB*, *hdc1*, *hdc2*, *tdc*, and *odc* genes. *Enterococcus durans* SJRP05 presented gelatinase as a virulence gene. The *gelE* gene may play an essential role in host tissue invasion because it encodes for an extracellular zinc endopeptidase capable of hydrolyzing gelatin, collagen, casein, and other small biologically active peptides, and it can therefore contribute to virulence; this gene seems to be located in the chromosome (Semedo et al., 2003; Ribeiro et al., 2014).

The presence of virulence factors in *Enterococcus* spp. has been reported in the literature. Semedo et al. (2003) evaluated the occurrence of several virulence traits in enterococcal strains, including food and clinical isolates. Based on their virulence factors, enterococcal strains were grouped into 3 main clusters and 10 subclusters. The results showed that virulence traits occur in enterococci irrespective of their origin (human or animal hosts, food, and environment) and species allocation, suggesting that such genetic determinants may be a common trait in the genus *Enterococcus*. In general, food, commensal, and environmental strains harbor fewer virulence determinants; therefore, a low risk is involved in their use in food fermentations. However, Semedo et al. (2003) recommend screening for virulence traits and their cross-synergies, particularly for commercial starters, probiotic strains, and products to be used by high-risk population groups. Perin et al. (2014) studied the virulence, antibiotic resistance, and biogenic amines of bacteriocinogenic lactococci and enterococci isolated from goat milk. Among the 18 strains of enterococci, only 2 (*E. durans* GEn02 and *E. durans* GEn03) did not present any virulence genes. Favaro et al. (2014) investigated the safety concern of virulence genes for nonstarter *E. faecium* strains isolated from cheese and the occurrence of virulence genes was limited. Chajęcka-Wierzchowska et al. (2016) reported that all of the enterococci isolated from retail shrimp carried between 5 and 9 tested virulence genes. The presence of *esp* (100%), *gelE* (88.6%), and *efaA* (77.1%) genes and sex pheromones *cpd* (100%), *cob* (94.3%), and *ccf* (94.3%) was more frequent in all of the tested

Table 5. Population (log cfu/mL; mean $\pm$ SD) of *Enterococcus durans* and *Enterococcus faecium* after simulation of the gastrointestinal tract

Strain	pH 2.0–2.5			pH 4.3–5.2 (240 min)	pH 6.7–7.5 (360 min)
	(0 min)	(60 min)	(120 min)		
<i>E. durans</i>					
SJRP17	8.85 $\pm$ 0.02 ^{A,a}	5.90 $\pm$ 0.04 ^{B,e}	5.50 $\pm$ 0.04 ^{C,d}	5.17 $\pm$ 0.10 ^{D,d}	5.14 $\pm$ 0.08 ^{D,d}
SJRP29	8.83 $\pm$ 0.06 ^{A,a}	7.43 $\pm$ 0.04 ^{B,c}	7.34 $\pm$ 0.03 ^{B,c}	7.35 $\pm$ 0.01 ^{B,b}	7.36 $\pm$ 0.05 ^{B,c}
<i>E. faecium</i>					
SJRP20	8.77 $\pm$ 0.03 ^{A,ab}	6.48 $\pm$ 0.04 ^{E,d}	7.97 $\pm$ 0.03 ^{B,b}	7.36 $\pm$ 0.05 ^{D,b}	7.69 $\pm$ 0.03 ^{C,b}
SJRP28	8.74 $\pm$ 0.02 ^{A,ab}	8.00 $\pm$ 0.01 ^{D,b}	8.71 $\pm$ 0.09 ^{AB,a}	8.38 $\pm$ 0.11 ^{C,a}	8.54 $\pm$ 0.05 ^{BC,a}
SJRP65	8.64 $\pm$ 0.01 ^{A,b}	8.68 $\pm$ 0.08 ^{A,a}	8.65 $\pm$ 0.03 ^{A,a}	5.38 $\pm$ 0.05 ^{C,c}	7.37 $\pm$ 0.10 ^{D,c}

^{A–E}Different letters in the same row indicate significant difference between the in vitro test analysis periods for the same strain ($P \leq 0.05$).

^{a–c}Different letters within the same column indicate significant difference among strains for the same period of in vitro test analysis ($P \leq 0.05$; $n = 3$).

enterococci. Hassan and Belal (2016) evaluated the antibiotic resistance and virulence genes in enterococcal strains isolated from hospitals' environment. They reported that a total of 57.1% of *E. faecium* and 50% of *E. durans* isolates were resistant to multiple drugs; however, only 3 isolates of *Enterococcus* spp. harbored resistance genes *ermB*, *tetL*, and *vanA*.

Lactic acid bacteria (especially *Enterococcus* spp.) can present virulence genes that, when expressed in food products, represent hazards for consumers (Perin et al., 2014). The absence of virulence factors, as we found, is noteworthy because it supports the safety of enterococcal strains. The identification of LAB strains with virulence factors is relevant, and epidemiological studies should be conducted to monitor the presence and dissemination of resistance and virulence genes from enterococci to establish public health measures (Tejedor Junco et al., 2015).

Thus, safety characteristics must be properly assessed during the evaluation of LAB strains for them to be considered starters or biopreservative cultures. Furthermore, the absence of virulence factors can be used when selecting virulence-free microorganisms for a future application as functional starter cultures in preparation of fermented milk products (Perin et al., 2014).

Assessment of Survival Under Simulated In Vitro GI Conditions

Considering the results of viability in the presence of medications, susceptibility to antimicrobial agents, and absence of virulence factors, the *E. durans* strains SJRP17 and SJRP29 and *E. faecium* strains SJRP20, SJRP28, and SJRP65 were selected for probiotic potential analysis by a simulation test of GI tract, and their behavior is shown in Table 5.

Enterococcus durans SJRP17 showed a decrease in its population in the very first 60 min of simulation, while *E. faecium* SJRP65 had a stable population only

during the simulation of the gastric phase. In general, viability loss occurs in the gastric phase because of its lower pH. In enteric phase I, pH increases and it generally promotes population increases (i.e., recovery of viability). The strains *E. durans* SJRP29 and *E. faecium* SJRP20 showed a decrease in their populations during the early stages of the gastric phase. Normally, the pH profile of H⁺-ATPase enzyme and cytoplasmic membrane of LAB are associated with their tolerance to acidic conditions, which vary among species as well as according to exogenous conditions, including growth medium and incubation conditions (Madureira et al., 2011). However, the strains maintained their populations during the simulation of the enteric phase I and enteric phase II, which means that they can possibly overcome the harsh conditions of the GI tract and survive; this survival is one criterion to be fulfilled for a strain to be considered potential probiotic bacteria. *Enterococcus faecium* SJRP28 was the most stable during the simulation of GI tract, presenting a higher final population (Table 5). At the end of the assay, except for *E. durans* SJRP17, all strains showed high viability (>7 log cfu/mL). Considering that probiotic food products must contain from 6 to 7 log cfu/mL or cfu/g to produce their beneficial effects in the body (Nazzaro et al., 2012), if such strains are stable under GI conditions when used in food systems, they will probably be able to resist to acid pH and high concentrations of bile salts. Consequently, they may potentially be used in functional products or supplements. However, it is necessary to take into account that a food matrix plays an essential role in the strains' viability.

Additionally, it is important to emphasize that the in vitro trials involving tolerance of pH and bile salts cannot predict patterns of behavior in the human body. After the consumption of some foods, the stomach pH (around 1.2–2.0) becomes 3.0 to 4.0, depending on the food. After ingesting yogurt and fermented milk, the stomach pH increases considerably and can reach 5.0 (Cheng et al., 2004). Furthermore, the tolerance of GI

transit has been found to vary among the strains (Vinderola and Reinheimer, 2003).

Tolerance to NaCl

The presence of high concentrations of NaCl (5.0 and 7.0%) was a limiting factor for the growth of *E. durans* SJRP29, whereas the incubation temperature had a more modest effect; however, the growth was slightly reduced at 37°C. Moreover, *E. durans* SJRP29 tolerated up to 3.0% NaCl at 30 and 37°C (Figure 1). Most LAB are halotolerant, which is an important characteristic for their use in dairy and fermented meat products (Penna et al., 2014), such as cheese and salami. Similar findings have been described in the literature. All LAB strains isolated from mozzarella cheese grew at 4% NaCl, while *E. faecalis*, *Lactobacillus garvieae*, *Lactococcus lactis*, and *Streptococcus thermophilus* showed absence of growth at 6.5 and 10.0% NaCl (Silva, 2015).

BSH Activity

Enterococcus durans SJRP29 was not able to hydrolyze both taurocholic and taurodeoxycholic acids. Similarly, none of the lactobacilli strains, including *L. casei*, *L. fermentum*, and *L. delbrueckii* ssp. *bulgaricus* tested by Casarotti et al. (2016) and *L. casei*, *L. fermentum*, *L. paracasei*, *L. plantarum*, and *L. pentosus* tested by Solieri et al. (2014) were able to hydrolyze both TDCA and TC. However, the BSH enzyme is produced by several microorganisms and is efficient in the hydrolysis of glycoconjugate and tauroconjugate bile salts, as reported by Nascimento (2015) for *L. fermentum* SJRP42 and *E. faecium* SJRP20. Moser and Savage (2001) observed that *L. buchneri* JCM1069 was able to hydrolyze bile salts of TDCA; however, it was not able to hydrolyze the salts of TC.

Auto-Aggregation, Co-Aggregation, and Hydrophobicity

The aggregation ability can help probiotic cultures to adhere to and colonize the GI tract and to modulate the immune system (Ouwehand and Vesterlund, 2004). *Enterococcus durans* SJRP29 presented a high value of aggregation at the temperatures of 37°C (63.04%) and 42°C (59.05%), which is positive because aggregation is an important feature for biofilm formation; however, aggregation was only 33.61% at 4°C. Similar results were observed for other LAB strains. *Leuconostoc mesenteroides* ssp. *mesenteroides* SJRP55 showed higher auto-aggregation at 30°C (85.64%), whereas when the tests were performed at 37°C and 5°C, auto-aggregation values were 51.05% and 51.67%, respectively (De Paula

Figure 1. Growth of *Enterococcus durans* SJRP29 in de Man, Rogosa, and Sharpe broth with 0% (◆), 0.5% (◇), 1.0% (■), 3.0% (□), 5.0% (▲), or 7.0% (△) NaCl at 30 and 37°C, shown as optical density (OD; 660 nm) measurements. The results are represented as an average of 9 readings.

et al., 2015a). In contrast, Todorov et al. (2011a) reported low values of auto-aggregation for LAB strains isolated from salmon, which ranged from 7.2% for *E. faecium* ET35 to 12.1% for *E. faecium* ET05.

Cell adhesion is a multistep process involving contact between the bacterial cell membrane and interacting surfaces. When adhesion occurs and the bacteria begin to colonize the GI tract, attachment of pathogens is prevented by blockage of the interaction with specific cell receptors or by inhibition of their fixation through enteric interactions (Kos et al., 2003).

Enterococcus durans SJRP29 also presented high values of co-aggregation with indicating microorganisms (Table 6). The co-aggregation between LAB and other cells, especially *Listeria monocytogenes*, may be considered a positive characteristic because it is one of the steps required for the elimination of undesirable strains from the GI tract (Todorov and Dicks, 2008). However, the strain did not have an inhibitory effect against the indicating microorganisms (data not shown). This characteristic would be desirable for a

Table 6. Auto-aggregation (%), co-aggregation (%), and cell surface hydrophobicity (%) of *Enterococcus durans* SJRP29 (n = 9)

Characteristic	Mean (% ± SD)
Auto-aggregation at 4°C	33.61 ± 0.34
Auto-aggregation at 37°C	63.04 ± 0.19
Auto-aggregation at 42°C	59.05 ± 0.45
Co-aggregation with <i>Lactobacillus fermentum</i> SJRP30	51.71 ± 0.16
Co-aggregation with <i>Lactobacillus casei</i> SJRP37	67.82 ± 0.19
Co-aggregation with <i>Listeria innocua</i> ATCC 33090	73.25 ± 0.33
Co-aggregation with <i>Listeria monocytogenes</i> ATCC 15313	44.01 ± 0.32
Hydrophobicity	96.53 ± 0.14
Mucin degradation	Negative

better competition between *E. durans* SJRP29 and pathogenic microorganisms that could be present in the diverse and complex microbiota of the human intestinal tract. Although no inhibitory activity was detected for *E. durans* SJRP29, the bacteria can compete with pathogenic microorganisms in several ways, such as for space or nutrients. Furthermore, LAB can improve the intestinal response, and when used in food production, they can improve sensory value and also be used as a strategy for food biopreservation (Kos et al., 2008).

In the present study, the selected strain showed a high level of cell surface hydrophobicity (96.53%, Table 6), measured using the interaction with n-hexadecane to simulate the ability to adhere to the intestinal epithelium. The observed result was higher than that reported in the literature for strains isolated from smoked salmon, previously identified as bacteriocin producers: *L. fermentum* (78.9%), *L. curvatus* (61.9–64.6%), *L. delbrueckii* (43.7%), *Pediococcus acidilactici* (51.3%), and *L. rhamnosus* GG (53.3%) (Todorov et al., 2011b); *Leuconostoc paramesenteroides* isolated from Cheddar cheese (46.11%) (Shobharani and Agrawal, 2011); and *Leuconostoc mesenteroides* ssp. *mesenteroides* SJRP55 (59.12%), isolated from mozzarella cheese (De Paula et al., 2015a). Additionally, cell surface hydrophobicity is strain specific, and the presence of different nutrients or carrier food matrices may affect the expression of adhesion genes in the microorganisms (Raghavendra and Halami, 2009).

Mucin Degradation

Enterococcus durans SJRP29 as well as *L. rhamnosus* GG (ATCC 53103) (negative control) did not show mucinolytic activity in either type of tested media [with or without 3% (wt/vol) of glucose] and was considered safe with regard to the maintenance of the mucosal integrity of the intestine. Conversely, *S. enterica* ssp. *enterica* serovar Typhimurium ATCC 14028 (positive control) was able to degrade mucin in vitro in a medium in which mucin was the only energy source (data not shown). These findings are in agreement with previous stud-

ies that investigated mucin degradation of *E. faecium* and *E. faecalis* bacteria (Abe et al., 2010). Any damage or disturbance of the mucin layer will compromise the defense function of the host mucosa (Zhou et al., 2001).

For the probiotic candidate *E. durans* SJRP29 to be used in functional food or supplements, it is essential to evaluate their additional characteristics, such as cytotoxicity and immunomodulatory properties. Furthermore, it is also important to carry out studies using in vitro dynamic models, such as simulator of the human intestinal microbial ecosystem, and in vivo tests before applying these strains to a food system.

CONCLUSIONS

Enterococcus faecium and *E. durans* both showed resistance to most of the tested medications but had substantial sensitivity to analgesics and antihypertensives; they also presented a wide susceptibility to antimicrobial agents. *Enterococcus durans* SJRP29 exhibited more resistance to the presence of medications than the probiotic *L. acidophilus* La-5. The strains submitted to an evaluation simulating the GI system expressed tolerance to pH and bile, with high viability. *Enterococcus durans* SJRP29 was selected for further evaluation and tolerated up to 3.0% NaCl at 30 and 37°C; it also presented good adhesion properties (high values of auto-aggregation, co-aggregation, and hydrophobicity). Additionally, the microorganism did not show BSH activity and mucin degradation. *Enterococcus durans* SJRP29 is considered safe and has good potential for application in functional food or supplements. These results encourage us to carry out additional tests to evaluate the probiotic features using in vitro dynamic models and in vivo tests before applying these strains to a food system.

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