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Evaluation of resistance genes and virulence factors in a food isolated *Enterococcus durans* with potential probiotic effect

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ABSTRACT

Enterococci belong to the lactic acid bacteria (LAB) group, which are often considered to provide benefit to the host organism when consumed. However, these microorganisms have a potential as infective agents, being necessary to evaluate the presence of virulence factors and resistance to antibiotics to warrant the safe use of new strains as probiotic cultures. This study aimed to detect genes of potential virulence factors related with adhesion, aggregation, biofilm formation and resistance to vancomycin, in addition to evaluate the antibiotic susceptibility and adhesion capacity of *Enterococcus durans* LA18s, a strain previously isolated from Minas Frescal cheese. The PCR reactions with specific primers to detect genes of adhesion collagen protein (*ace*), aggregation substances (*agg* and *asa*), *bopA* (putative glycosyltransferase), *bopB* (beta-phosphoglucomutase), *bopC* (aldose 1-epimerase), and *bopD* (sugar-binding transcriptional regulator) were negative for *E. durans* LAB18s. In addition, the strain did not present the resistance genes *vanA*, *vanC1* and *vanC2/3*, and exhibited sensibility to antibiotics commonly used in animal feed, such as erythromycin, tetracycline, vancomycin, gentamicin and penicillin. This strain also showed a strong capacity of biofilm formation and exhibited satisfactory auto-aggregative and hydrophobicity features. The results suggest that this strain can be safely used in animal feed.

1. Introduction

The genus *Enterococcus* belongs to the group of lactic acid bacteria (LAB). Despite enterococci are ubiquitous bacteria, their primary habitat is the intestine of health warm-blooded animals. In this complex ecosystem, there is a delicate balance between the gut microbiota and the host. *Enterococcus faecalis*, and in a lesser extent *Enterococcus faecium*, are the predominant species in the intestine (Cebrián et al., 2012; Foulquié-Moreno, Sarantinopoulos, Tsakalidou, & de Vuyst, 2006). Enterococci may play beneficial roles in various traditional food products as they contribute to the ripening and aroma development of certain cheeses or fermented sausages (Franz, Huch, Abriouel, Holzapfel, & Gálvez, 2011; Giraffa, 2002).

Certain enterococcal strains are also successfully used as probiotics to improve human or animal health. These bacteria ingested in high numbers may achieve functional or probiotic effects especially for treatment of diseases such as irritable bowel syndrome, diarrhea or antibiotic associated diarrhea, and for health improvement such as lowering cholesterol levels or immune regulation (Franz et al., 2011). As the site of activity is the gastrointestinal system, these beneficial effects are brought about by the interaction of the enterococci with the gut microbiota. Likewise, the use of enterococci in animal feed has similar goals, i.e. either to prevent disease by influencing the gastrointestinal micropopulations or stimulation of the immune system (Gaggìa, Mattarelli, & Biavati, 2010; Kreuzer et al., 2012).

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On the other hand, other enterococcal strains are associated with nosocomial infections and cause human diseases such as endocarditis, bacteremia, and infections of the urinary tract, central nervous system, abdomen, and pelvis (Foulquié-Moreno et al., 2006). Such pathogenic strains often carry multiple antibiotic resistances and virulence factors such as adhesins and hemolysins. The role of some enterococci in human disease raises concerns for their safe use as either starters in food production or as probiotics. Studies on the incidence of virulence factors have shown that isolates from food can also present virulence factors and antibiotic resistance (Franz et al., 2001; Trivedi, Cupakova, & Karpiskova, 2011). Generally, the occurrence of virulence determinants appears to be higher in *E. faecalis* strains than in *E. faecium* strains of food origin (Cebrián et al., 2012; Leavis et al., 2004).

The *Enterococcus* strain evaluated in this study was isolated form a typical Brazilian cheese, and has being investigate for its capability for selenium bioaccumulation (Pieniz, Andreazza, Pereira, Camargo, & Brandelli, 2013), and probiotic potential for possible application as enriched inoculum in animal feed (Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli, 2014). However, to guarantee the safe use of this strain as probiotic culture it is also necessary to evaluate the presence of potential virulence factors and resistance to antibiotics. Thus, the aim of this study was to detect genes involved in adhesion, aggregation, biofilm formation, resistance genes to vancomycin and susceptibility for antibiotics, as well as to evaluate the adhesion capacity of this strain.

2. Materials and methods

2.1. Microorganism

The strain *Enterococcus durans* LAB18s was isolated from Minas Frescal cheese (typical Brazilian soft cheese), and characterized as describe elsewhere (Pieniz et al., 2013). The strain was maintained in stock culture at −20 °C in Brain Heart Infusion (BHI; Oxoid) containing 20% (v/v) glycerol.

2.2. Antibiotic susceptibility test

The antibiotic susceptibility testing was performed according to standard disc diffusion method recommended by the Clinical and Laboratory Standards Institute (CLSI, 2008). The isolate was inoculated onto Mueller-Hinton (MH) agar plates and incubated at 35 °C for 24 h. After growth, colonies were suspended in sterile saline solution (9 g/L NaCl) and adjusted to an OD_{600} 0.150 ± 0.02, which corresponds to 0.5 McFarland scale. Then, using swabs, plates with MH agar were inoculated with the standardized solution. Five antibiotics commonly used in animal feed were tested: erythromycin-15 (ERI), tetracycline-30 (TET), vancomycin-30 (VAN), gentamicin-120 (GEN) and penicillin-10 (PEN). The minimal inhibitory concentration (MIC) for vancomycin was determined using the E test® method according to the manufacturer's instructions. The diameter of inhibition zones were measured after incubation for 24 h at 35 °C. The isolate was classified according to the CLSI criteria as susceptible, intermediate or resistant to the antibiotic (CLSI, 2008). Data were expressed in millimeters (mm) of inhibition zone. The experiment was performed in triplicate in three independent experiments.

2.3. DNA extraction

The strain was grown in BHI medium at 35 °C for 24 h. Subsequently, colony forming units of bacterial cells were collected and then DNA was extracted using the kit Promega Wizard Genomic DNA Purification Kit™ (Cat # A1125).

2.4. Detection of virulence and resistance genes

The strain *E. durans* LAB18s was analyzed for the presence of virulence genes *ace* (adhesion collagen protein), *agg* (aggregation), *asa* (aggregation), *bopA*, *bopB*, *bopC* and *bopD* (biofilm formation). The polymerase chain reaction (PCR) was performed in a total volume of 25 μL containing: 2 μL DNA template, 1.5 mM $MgCl_2$, 10 mM primers, 200 mM each dNTP, 1 unit (U) of DNA Taq Polymerase and 1× reaction buffer.

The strain was also evaluated for resistance genes *vanA*, *vanC*1 and *vanC2/3* by PCR. The PCR reaction was performed in a total volume of 25 μL as described above, excepting the concentration of $MgCl_2$ was 2 mM.

The primer sequences are described in Table 1. The PCR conditions for all genes were denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 1 min, annealing extension according to Table 1, followed by final extension at 72 °C for 10 min. The PCR products were analyzed by electrophoresis with 1.5% agarose gels, stained with 5% (w/v) ethidium bromide solution and visualized under ultraviolet light.

2.5. Plasmid DNA profile

Plasmid DNA was extracted with a small-scale alkaline lysis method (Sambrook, Maniatis, & Fritsch, 1989). Extracted plasmids were electrophoresed for 2 h in a horizontal 0.7% agarose gel with pH 8.0 Tris–EDTA (TE) buffer. The gels were stained with ethidium bromide 0.5 μg/mL for 20 min, and bands were visualized by UV transilluminator. Molecular weight marker KAPA Universal Ladder was used as the DNA standard marker. *Enterococcus mundtii* J5 isolated from feces and a commercial strain of *Escherichia coli* (NEB 10-beta competent; New England BioLabs, Ipswich, MA, USA) containing a 10 Kb plasmid were used for comparison.

2.6. Evaluation of biofilm formation capability

The isolate was analyzed for its ability of biofilm formation as described by Stepanovic, Vukovic, Dakic, Savic, and Svabic-Vlahovic

Table 1
PCR primers and the annealing temperatures used to detect the virulence genes in *Enterococcus* spp.

Primer	Sequence (5′-3′)	Product (bp)	AT[a] (°C)	Reference
ace f	AAAGTAGAATTAGATCACAC	320	48	Duprè, Zanetti, Schito, Fadda, & Sechi, 2003
ace r	TCTATCACATTCGGTTGCG			
agg f	AAGAAAAAGTAGACCAAC	1553	48	Eaton & Gasson, 2001
agg r	AACGGCAAGACAAGTAAATA			
asa f	GATACAAAGCCAATGTGGTTCCT	101	48	Dunny, Craig, Carron, & Clewell, 1979
asa r	TAAAGAGTCGCCACGTTTCACA			
bopA f	CAGCGACATGGACAGCCTAC	108	48	Vebø, Snipen, Nes, & Brede, 2009
bopA r	TTGCAGGACCGTCGAGTAAA			
bopB f	ATGACAGAATCCAAAACTGC	687	48	Cassenego, 2014
bopB r	TTACGAAGGGGTTGATTCAC			
bopC f	TTATAGAAGGTTAAATTGAT	1010	48	Cassenego, 2014
bopC r	ATGAAGGATAATCGTATCAC			
bopD f	GGCTTCCTCGTTGATGGCTTC	126	48	Hufnagel et al. 2004
bopD r	ACGGCACGGAATTTGGGTAAAC			
vanA f	GGGAAAACGACAATTGC	732	50	Depardieu, Perichon, & Courvalin, 2004
vanA r	GTACAATGCGGCCGTTA			
vanC1 f	GGTATCAAGGAAACCTC	822	54	Dutka-Malen, Evers, & Courvalin, 1995
vanC1 r	CTTCCGCCATCATAGCT			
vanC2/3 f	CGGGGAAGATGGCAGTAT	848	54	Satake, Clark, Rimland, Nolte, & Tenover, 1997
vanC2/3 r	CGCAGGGACGGTGATTTT			

[a] AT = annealing temperature.

(2000). The strain was previously inoculated in a plate containing BHA and incubated at 35 °C for 24 h. Microtitration plates were filled with 180 μL of sterile BHI. After overnight growth, colonies were suspended in sterile saline and the OD_{600} was adjusted to 0.150 ± 0.02. Then, 20 μL of this suspension were inoculated in each well containing 180 μL BHI. *Staphylococcus aureus* ATCC 25923 was used as a positive control, and a negative control was performed with BHI alone. The plates were covered and incubated at 35 °C for 24 h. After growth, the cultures were aspirated with a multichannel pipette and the wells were washed three times with 200 μL saline solution. The micro-plate was inverted onto absorbent paper to dry and subsequently the samples were fixed with 150 μL of methanol (CH_3OH) for 20 min. After this time, methanol was discarded and the plates were maintained inverted overnight. The samples were stained with 150 μL crystal violet (5 g/L) for 15 min. After the plates were inverted and excess was removed under running water. Then, after a short drying period 150 μL ethanol (95% v/v) was added. The plates were maintained for 30 min and then the absorbance was measured with microplate reader (Anthos 2010 17550 Type 4894) at 450 nm.

Based on the OD produced by bacterial films, strains were classified into the following categories: no biofilm producers (0), weak (+), moderate (++) or strong biofilm producers (+++), as previously described (Stepanovic et al., 2000). Briefly, the cut-off OD (ODc) was defined as three standard deviations above the mean OD of the negative control. Strains were classified as follows: OD ≤ ODc = no biofilm producer, ODc < OD ≤ (2 × ODc) = weak biofilm producer, (2 × ODc) < OD ≤ (4 × ODc) = moderate biofilm producer and (4 × ODc) < OD = strong biofilm producer. All tests were carried out in triplicate.

2.7. Determination of cell surface hydrophobicity

The surface hydrophobicity was determined *in vitro* according to Rosenberg, Gutnick, and Rosenberg (1980) with some modifications. Briefly, the isolate was inoculated in BHI and incubated at 35 °C. After 24 h, the cells were collected by centrifugation at 10,000 × g for 10 min at 4 °C, washed twice and suspended in 10 mM phosphate buffered saline (PBS) pH 7.2, and the OD_{600} was adjusted to 0.800 ± 0.05 (A_0). An aliquot of 0.6 mL of either xylene or chloroform was added to tubes containing 3 mL of bacterial suspension. The solution was homogenized with vortex for 2 min. The tubes were allowed to stand at 35 °C for 2 h for phase separation. After this period, the aqueous phase (A) was carefully removed and the OD_{600} was measured. The cell surface hydrophobicity was calculated from three replicates using the following equation: $H\% = [((A_0 - A)/A_0) \times 100]$.

Two solvents were evaluated in this study. The xylene and chloroform were used as apolar and acid monopolar solvents, respectively. In terms of bacterial adhesion, only xylene reflects the cell surface hydrophobicity. The values obtained with chloroform were considered as an electron acceptor characteristic of the bacteria (Bellon-Fontaine, Rault, & Van Oss, 1996).

2.8. Auto-aggregation

The auto-aggregation was evaluated according to Juárez-Tomás, Wiese, and Nader-Macías (2005) with some modifications. The strain was grown in BHI at 35 °C for 24 h. After this, cells were sampled by centrifugation at 10,000 × g for 10 min at 4 °C, washed twice and suspended in 10 mM PBS (pH 7.2). The OD_{600} was adjusted to 0.600 ± 0.05 (A_0). The cell suspension was incubated at 35 °C, and the ability to auto-aggregation was measured by OD_{600} measurement after 1–24 h (At). The auto-aggregation was determined using the following equation: $\%\ AA = [((A_0 - A_t)/A_0) \times 100]$.

2.9. DNase test

DNase activity was tested as described by Bannerman (2003) using the medium DNase Test Agar with toluidine blue (Himedia, São Paulo, Brazil). A clear halo around the colonies was indicative of a positive result.

2.10. Hemolytic activity

The hemolytic activity was evaluated according Foulquié-Moreno, Callewaert, Devreese, van Beeumen, & de Vuyst (2003). The strain was tested for hemolytic activity using blood agar (7% v/v sheep blood) for 48 h incubation at 37 °C. Strains that produced green-hued zones around the colonies (α-hemolysis) or did not produce any effect on the blood plates (γ-hemolysis) are considered non hemolytic. Strains displaying blood lyses zones around the colonies are classified as hemolytic (β-hemolysis).

3. Results

3.1. Virulence genes

The presence of virulence genes was investigated by PCR. The genes for adhesion collagen protein (*ace*), and aggregation substances (*agg* and *asa*) were not detected in *E. durans* LAB18s, whereas the expected PCR products were observed for *E. faecalis* ID 2389 used as a positive control (Fig. 1). The genes *bopA* (putative glycosyltransferase), *bopB* (beta-phosphoglucomutase), *bopC* (aldose 1-epimerase), and *bopD* (sugar-binding transcriptional regulator) involved in the metabolism of maltose and biofilm formation were also evaluated. The strain *E. durans* LAB18s did not present any PCR product for the virulence factors evaluated (Fig. 1).

Likewise, *E. durans* LAB18s was evaluated for resistance genes *vanA*, *vanC1* and *vanC2/3*. It can be observed in Fig. 2 that these resistance genes were not present in *E. durans* LAB18s.

A plasmid DNA extraction was performed using standard miniprep methods and the results from the plasmid DNA profile showed the absence of plasmids in the strain LAB18s (Fig. 3).

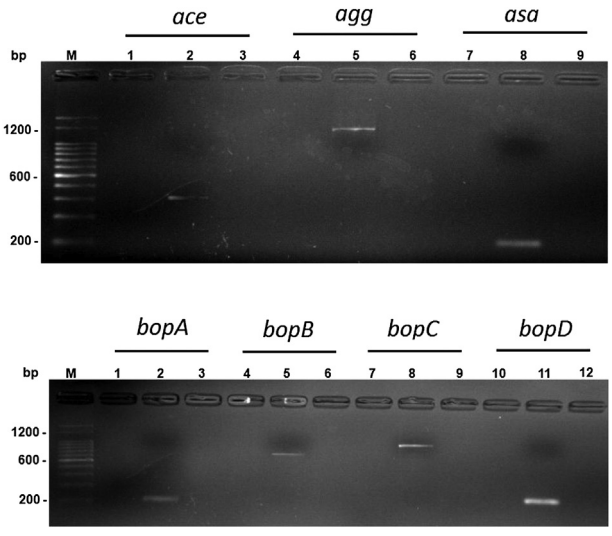

Fig. 1. Amplification of virulence-associated genes *ace*, *agg* and *asa* (upper panel), *bopA*, *bopB*, *bopC* and *bopD* (lower panel) by PCR. The letter bp indicates bases pair; the letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen) and the numbers are the respective samples: (1, 4, 7, 10) negative controls; (2, 5, 8, 11) positive controls; (3, 6, 9, 12) the absence of the genes in *E. durans* LAB18s. Vancomycin-resistant *Enterococcus faecalis* ID 2389 was used as positive control.

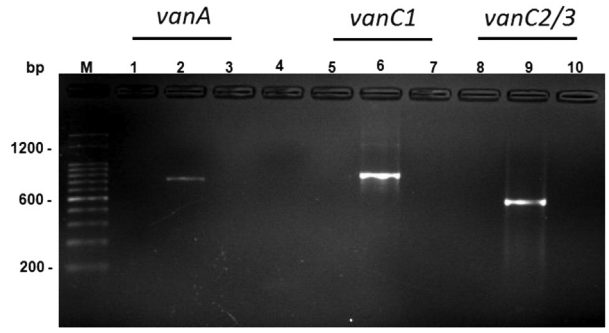

Fig. 2. Amplification of resistance-associated genes *vanA*, *vanC1* and *vanC2/3* by PCR. The letter bp indicates bases pair; the letter M indicates the molecular size marker (100 bp DNA Ladder, Invitrogen) and the numbers represent: (1, 5, 8) negative controls; (2, 6, 9) positive controls; (3, 7, 10) absence of *vanA*, *vanC1* and *vanC2/3* gene, respectively, in *E. durans* LAB18s. Vancomycin-resistant *Enterococcus faecalis* ID 2389 was used as positive control to *vanA*, and *Enterococcus faecalis* CB356 was used as positive control to *vanC1* and *vanC2/3*.

3.2. Antibiotic susceptibility

Analysis of the results obtained for antibiotic susceptibility test showed that the isolate exhibited high sensibility for all antibiotics tested, according to CLSI standards (Table 2). When the isolate *E. durans* LAB18s was analyzed by E test®, a MIC ≤4 μg/mL (0.38 μg/mL) was observed indicating the susceptibility of the strain to vancomycin.

3.3. Biofilm formation, hydrophobicity and auto-aggregation

The strain showed elevated adherence to microplates and was classified as strong biofilm producer (Stepanovic et al., 2000). Interestingly, this factor was not associated with the presence of virulence-associated genes concerning biofilm formation. This capacity could be specifically related with the adhesion properties of *E. durans* LAB18s.

The hydrophobicity of *E. durans* LAB18s was analyzed using two solvents: xylene, a non-polar solvent; and chloroform, an acidic monopolar solvent. The results of hydrophobicity and acid character of the cell wall was expressed in percentage, and the values were 31.1 ± 0.01% and 66.8 ± 0.02% for xylene and chloroform, respectively. The strain showed a cell surface more acidic (chloroform) when compared with the values for hydrophobicity observed using xylene.

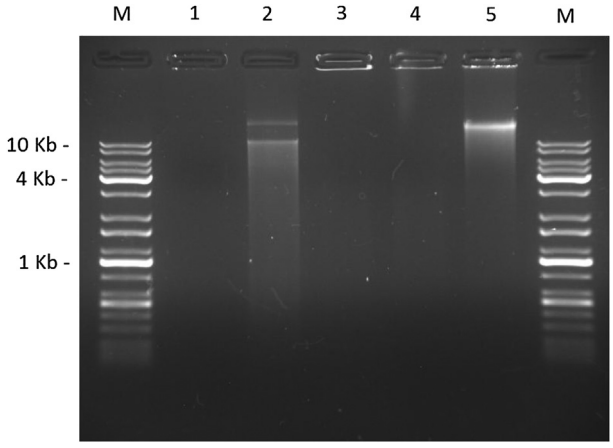

Fig. 3. Plasmid DNA profile. Plasmid DNA extracted from *E. coli* NEB 10-beta (2), *E. mundtii* J5 (3) and *E. durans* LAB18s (4) were analyzed in 0.7% agarose gels. (1) negative control, (5) chromosomal DNA of *E. durans* LAB18s, (M) molecular weight marker KAPA Universal Ladder.

Table 2
Antibiotic susceptibility of *E. durans* LAB18s and classification according to Clinical Laboratory Standards Institute (CLSI).

Antibiotic		CLSI classification	
	Inhibition zone (mm)[a]	Susceptible zone (mm)	S–I–R[b]
Erythromycin	23.3 ± 0.35	≥23	S
Tetracycline	20.5 ± 0.18	≥19	S
Vancomycin	19.0 ± 0.47	≥17	S
Gentamicin	21.5 ± 0.21	≥10	S
Penicillin	26.0 ± 0.47	≥15	S

[a] Values are expressed as mean ± standard error of three independent experiments.
[b] Standard interpretation of antimicrobial susceptibility tests of enterococci with disc diffusion method in accordance to CLSI standards (CLSI, 2008): S, susceptible; I, intermediate; R, resistant.

The percentage of auto-aggregation increased exponentially in the initial incubation period, from 27% at 1 h to 47% at 4 h, and reaching values of 79% at 24 h of incubation (Fig. 4). These data indicate that the strain has auto-aggregative and hydrophobicity features and these could be related with adherence capability.

The isolate *E. durans* LAB18s did not exhibit any hemolytic effect (γ-hemolysis, α-hemolysis and/or β-hemolysis) after 48 h incubation in blood agar plates (Fig. S1). It was also observed that the DNAse test exhibited negative results (Fig. S1).

4. Discussion

Enterococci are present in numerous food and fermented dairy products, and the species *E. faecalis*, *E. faecium*, and *E. durans* are commonly found in milk products (Franz et al., 2011). The presence of enterococci in foods has been known for a long time, but only recently they have been considered as potential probiotic agents (Ogier & Serror, 2008). Thus, obtaining precise information about these microorganisms, and to promote studies for possible application in food or feed is mandatory to allow their safe use. The results from this study suggest that the strain *E. durans* LAB18s may be used as a feed additive in future animal testing.

Antibiotic susceptibility of *E. durans* LAB18s was evaluated. The wide use of tetracycline in animal husbandry is frequently associated with the high levels of tetracycline resistance found among enterococci (Hayes, English, Carr, Wagner, & Joseph, 2004). In contrast, the isolate *E. durans* LAB18s was sensitive to this antibiotic.

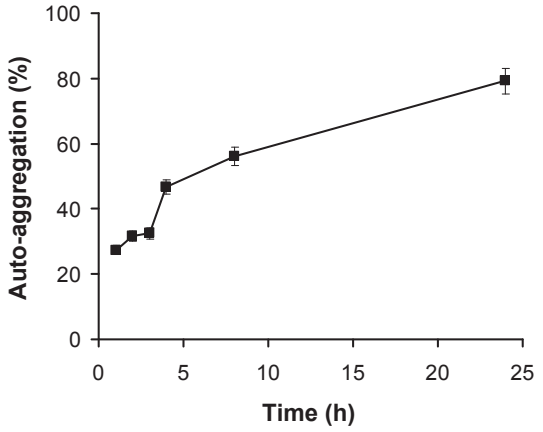

Fig. 4. Auto-aggregation of *E. durans* LAB18s after incubation at 35 °C. Data were expressed as mean percentage (%) ± standard error of three independent experiments.

Likewise, *E. durans* LAB18s was susceptible to erythromycin according to CLSI classification. Resistance to erythromycin, a representative of the macrolide antibiotics, would be a matter of concern because macrolides are common substitutes for individuals with penicillin allergy (Peters, Mac, Wichmann-Schauer, Klein, & Ellerbroek, 2003). The resistance of enterococci to β-lactam antibiotics seems to be associated with clinical strains and often inaccurately generalized in the literature to the genus (Kak & Chow, 2002). However, the results obtained in the present study are not in agreement with this generalization, since the isolate was susceptible to penicillin, in agreement with results described by other authors (McGowan-Spicer, Fedorka-Cray, Frye, & Meinersmann, 2008; Peters et al., 2003). Vancomycin-resistant enterococci (VRE) have emerged in the last decade as a frequent cause of nosocomial infections, and the presence of VRE has been detected among meat isolates (Messi, Guerrieri, Niederhäusern, Sabia, & Bondi, 2006). However, the isolate *E. durans* LAB18s was not resistant to vancomycin, exhibiting sensitivity in both antibiogram and E-test®.

Besides the analyses of vancomycin susceptibility, *E. durans* LAB18s was evaluated for the presence of vancomycin resistance genes. The genes *vanA*, *vanC1* and *vanC2/3* were investigated by PCR resulting in negative amplification for any of the tested genes. The assessment of resistance genes to vancomycin in strains with possible use in animal feed is of utmost importance, since this antibiotic is not metabolized by the animals and remains in the active form in the intestines (Yap et al., 2008), promoting VRE. In Europe, VRE (predominantly the *vanA* genotype) are frequently isolated in non-hospitalized patients, animals and environmental sources, and they are rare in the hospital setting (Bonten, Willems, & Weistein, 2001). This suggests that these bacteria come from a community source; they could be part of the normal animal microbiota and passed through the food chain.

Depending on the type and combination of virulence factors, they become crucial for the strain pathogenicity. In *Enterococcus* species, genes conferring antibiotic resistance and other virulence factors, such as aggregation substance (*agg* and *asa*) and collagen adhesins (*ace*), can be acquired by genetic exchange (Eaton & Gasson, 2001; Mundy, Sahm, & Gilmore, 2000). We analyzed the presence of *ace*, *agg* and *asa* by PCR amplification and none of these virulence genes were detected in *E. durans* LAB18s. Aggregation substance is a pheromone-inducible surface protein of *E. faecalis* that promotes mating aggregate formation during bacterial conjugation (Mundy et al., 2000). As an important component of the bacterial pheromone-responsive genetic exchange system, aggregation substance mediates efficient enterococci donor-recipient contact to facilitate plasmid transfer. Likewise, aggregation substance increases the hydrophobicity of the enterococcal surface, which may prevent or delay phagocytosis and the subsequent fate of the organism.

Creti, Koch, Fabretti, Baldassarri, and Huebner (2006) identified the operon *bopABCD* involved in biofilm formation in *E. faecalis*, which appears to be regulated by the Fsr system through quorum-sensing. In this study, the virulence genes *bopA*, *bopB*, *bopC* and *bopD* were also analyzed by PCR amplification, but *E. durans* LAB18s did not exhibit these genes. Biofilm production plays a major role in the pathogenesis of many important microbes and the occurrence of biofilms in enterococcal infections has been frequently reported (Mohamed & Huang, 2007). Hufnagel, Koch, Creti, Baldassarri, and Huebner (2004) reported that production of biofilm may be a virulence factor in enterococci that leads to prolonged bacteremia. The ability of the strain tested to produce biofilm *in vitro* was correlated with its persistence in the mouse bacteremia model *in vivo*. In the same way, the *in vitro* biofilm formation capability was analyzed in this study showing that *E. durans* LAB18s exhibited a strong capacity of biofilm formation. However, this capacity was not associated with the virulence-associated genes. Probably this finding is related with the adhesion properties, a desirable characteristic of probiotic strains (Samot, Lebreton, & Badet, 2011).

In this study, the hydrophobicity and auto-aggregation capacity of *E. durans* LAB18s was also evaluated, and the strain showed satisfactory adhesion properties. Hydrophobicity and auto-aggregation tests are based on the principle that attachment to the epithelial surface is a necessary first step for the colonization of probiotic microorganisms (Ocaña & Nader-Macías, 2002; Vinderola & Reinheimer, 2003). The hydrophobicity is related to the hydrophobic components present in the outer membrane of the organism and it is believed that hydrophobic interactions play an important role in the adhesion of bacteria to epithelial cells. Although the test is performed with solvents, it is possible to assess qualitatively the bacterial surface as either polar or non-polar, indicating the potential for adhesion to non-polar surfaces such as the cell surfaces (Ocaña & Nader-Macías, 2002; Rosenberg et al., 1980). Bacterial aggregation between microorganisms of the same strain (auto-aggregation) is considered important in several ecological niches (Del Re, Sgorbati, Miglioli, & Palenzona, 2000).

In summary, the strain *E. durans* LAB18s is a strain that might be considered safe for consumption, because it does not show virulence factors or vancomycin resistance genes when tested for *ace*, *agg*, *asa*, *bopA*, *bopB*, *bopC*, *bopD*, *vanA*, *vanC1* and *vanC2/3*. Furthermore, the isolate was susceptible to all antibiotics tested, and also showed adhesion capacity. In addition to its capability to bioaccumulate selenium, *E. durans* LAB18s may be used as selenium-enriched culture to be added in animal feed.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.foodcont.2014.11.012.

References

Bannerman, T. M. (2003). *Staphylococcus, Micrococcus* and other catalase-positive cocci that grow aerobically. In P. R. Murray (Ed.), *Manual of clinical microbiology* (8th ed.). (pp. 384–404). Washington, DC: ASM Press.

Bellon-Fontaine, M. N., Rault, J., & Van Oss, C. J. (1996). Microbial adhesion to solvents: a novel method to determine the electron donor/electron-acceptor or Lewis acid-base properties of microbial cells. *Colloids and Surfaces B: Biointerfaces, 7*, 47–53.

Bonten, M. J. M., Willems, R., & Weistein, R. A. (2001). Vancomycin-resistant enterococci: why are they here, and where do they come from? *Lancet Infectious Diseases, 1*, 314–325.

Cassenego, A. P. V. (2014). *Analysis of virulence factors in Enterococcus faecalis isolated from humans, foods and chickens* (Ph.D. Thesis) (p. 127). Porto Alegre, Brazil: Universidade Federal do Rio Grande do Sul.

Cebrián, R., Baños, R. A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., & Maqueda, M. (2012). Characterization of functional, safety, and probiotic properties of *Enterococcus faecalis* UGRA10, a new AS-48-producer strain. *Food Microbiology, 30*, 59–67.

CLSI. (2008). Vol. 28, No. 8 (3rd ed.). *M31-A3. Performance standards for antimicrobial disc and dilution susceptibility tests for bacteria isolated from animals; approved standard* (pp. 1–99). Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute.

Creti, R., Koch, S., Fabretti, F., Baldassarri, L., & Huebner, J. (2006). Enterococcal colonization of the gastro-intestinal tract: role of biofilm and environmental oligosaccharides. *BMC Microbiology, 6*, 1–8.

Del Re, B., Sgorbati, B., Miglioli, M., & Palenzona, D. (2000). Adhesion, autoaggregation and hydrophobicity of 13 strains of *Bifidobacterium longum*. *Letters in Applied Microbiology, 31*, 438–442.

Depardieu, F., Perichon, B., & Courvalin, P. (2004). Detection of the van alphabet and identification of enterococci and staphylococci at the species level by multiplex PCR. *Journal of Clinical Microbiology, 42*, 5857–5860.

Dunny, G. M., Craig, R. A., Carron, R. L., & Clewell, D. B. (1979). Plasmid transfer in *Streptococcus faecalis*: production of multiple sex pheromones by recipients. *Plasmid, 2*, 454–465.

Duprè, I., Zanetti, S., Schito, A. M., Fadda, G., & Sechi, L. A. (2003). Incidence of virulence determinants in clinical *Enterococcus faecium* and *Enterococcus faecalis* isolates collected in Sardinia (Italy). *Journal of Medical Microbiology, 52*, 491–498.

Dutka-Malen, S., Evers, S., & Courvalin, P. (1995). Detection of glycopeptide resistance genotypes and identification to the species level of clinically relevant enterococci by PCR. *Journal of Clinical Microbiology, 33*, 24–27.

Eaton, T. J., & Gasson, M. J. (2001). Molecular screening of *Enterococcus* virulence determinants and potential for genetic exchange between food and medical isolates. *Applied and Environmental Microbiology, 67*, 1628–1635.

Foulquié-Moreno, M. R., Callewaert, R., Deberse, B., van Beeumen, J., & de Vuyst, L. (2003). Isolation and biochemical characterisation of enterocins produced by enterococci from different sources. *Journal of Applied Microbiology, 94*, 214–229.

Foulquié-Moreno, M. R., Sarantinopoulos, P., Tsakalidou, E., & de Vuyst, L. (2006). The role and application of enterococci in food and health. *International Journal of Food Microbiology, 106*, 1–24.

Franz, C. M., Huch, M., Abriouel, H., Holzapfel, W. H., & Gálvez, A. (2011). Enterococci as probiotics and their implications in food safety. *International Journal of Food Microbiology, 151*, 125–140.

Franz, C. M. A. P., Muscholl-Silberhorn, A. B., Yousif, N. M. K., Vancanneyt, M., Swings, J., & Holzapfel, W. H. (2001). Incidence of virulence factors and antibiotic resistance among enterococci isolated from food. *Applied and Environmental Microbiology, 67*, 4385–4389.

Gaggìa, F., Mattarelli, P., & Biavati, B. (2010). Probiotics and prebiotics in animal feeding for safe food production. *International Journal of Food Microbiology, 141*, S15–S28.

Giraffa, G. (2002). Enterococci from foods. *FEMS Microbiology Reviews, 26*, 163–171.

Hayes, J. R., English, L. L., Carr, L. E., Wagner, D. D., & Joseph, S. W. (2004). Multiple-antibiotic resistance of *Enterococcus* spp. isolated from commercial poultry production environments. *Applied and Environmental Microbiology, 70*, 6005–6011.

Hufnagel, M., Koch, S., Creti, R., Baldassarri, L., & Huebner, J. (2004). A putative sugar-binding transcriptional regulator in a novel gene locus in *Enterococcus faecalis* contributes to production of biofilm and prolonged bacteremia in mice. *Journal of Infectious Diseases, 189*, 420–430.

Juárez-Tomás, M. S., Wiese, B., & Nader-Macías, M. E. (2005). Effects of culture conditions on the growth and auto-aggregation ability of vaginal *Lactobacillus johnsonii* CRL 1294. *Journal of Applied Microbiology, 99*, 1383–1391.

Kak, V., & Chow, J. W. (2002). Acquired antibiotic resistances in enterococci. In M. S. Gilmore (Ed.), *The enterococci: Pathogenesis, molecular biology and antibiotic resistance* (pp. 355–383). Washington: American Society for Microbiology.

Kreuzer, S., Machanowska, P., Aβmus, J., Sieber, M., Pieper, R., Schmidt, M. F. G., et al. (2012). Feeding of the probiotic bacterium *Enterococcus faecium* NCIMB 10415 differentially affects shedding of enteric viruses in pigs. *Veterinary Research, 43*, 58,. http://dx.doi.org/10.1186/1297-9716-43-58.

Leavis, H., Top, J., Shankar, N., Borgen, K., Bonten, M., van Embden, J., et al. (2004). A novel putative enterococcal pathogenicity island linked to the *esp* virulence gene of *Enterococcus faecium* and associated with epidemicity. *Journal of Bacteriology, 186*, 672–682.

McGowan-Spicer, L. L., Fedorka-Cray, P. J., Frye, J. G., & Meinersmann, R. J. (2008). Antimicrobial resistance and virulence of *Enterococcus faecalis* isolated from retail food. *Journal of Food Protection, 71*, 760–769.

Messi, P., Guerrieri, E., Niederhäusern, S., Sabia, C., & Bondi, M. (2006). Vancomycin-resistant enterococci (VRE) in meat and environmental samples. *International Journal of Food Microbiology, 107*, 218–222.

Mohamed, J. A., & Huang, D. B. (2007). Biofilm formation by enterococci. *Journal of Medical Microbiology, 56*, 1581–1588.

Mundy, L. M., Sahm, D. F., & Gilmore, M. (2000). Relationships between enterococcal virulence and antimicrobial resistance. *Clinical Microbiology Reviews, 13*, 513–522.

Ocaña, V., & Nader-Macías, M. E. (2002). Vaginal lactobacilli: self- and co-aggregating ability. *British Journal of Biomedical Sciences, 59*, 183–190.

Ogier, J., & Serror, P. (2008). Safety assessment of dairy microorganisms: the *Enterococcus* genus. *International Journal of Food Microbiology, 126*, 291–301.

Peters, J., Mac, K., Wichmann-Schauer, H., Klein, G., & Ellerbroek, L. (2003). Species distribution and antibiotic resistance patterns of enterococci isolated from food of animal origin in Germany. *International Journal of Food Microbiology, 88*, 311–314.

Pieniz, S., Andreazza, R., Anghinoni, T., Camargo, F. A. O., & Brandelli, A. (2014). Probiotic potential, antimicrobial and antioxidant activities of *Enterococcus durans*. *Food Control, 37*, 251–256.

Pieniz, S., Andreazza, R., Pereira, J. Q., Camargo, F. A. O., & Brandelli, A. (2013). Production of selenium enriched biomass by *Enterococcus durans*. *Biological Trace Element Research, 155*, 447–454.

Rosenberg, M., Gutnick, D., & Rosenberg, E. (1980). Adherence of bacteria to hydrocarbons: a simple method for measuring cell surface hydrophobicity. *FEMS Microbiology Letters, 9*, 29–33.

Sambrook, J., Maniatis, T., & Fritsch, E. F. (1989). *Molecular cloning: A laboratory manual* (2nd ed.). (p. 1626). Cold Spring Harbor, New York: Cold Spring Harbor Laboratory.

Samot, J., Lebreton, J., & Badet, C. (2011). Adherence capacities of oral lactobacilli for potential probiotic purposes. *Anaerobe, 17*, 69–72.

Satake, S., Clark, N., Rimland, D., Nolte, F. S., & Tenover, F. C. (1997). Detection of vancomycin-resistant enterococci in fecal samples by PCR. *Journal of Clinical Microbiology, 35*, 2325–2330.

Stepanovic, S., Vukovic, D., Dakic, I., Savic, B., & Svabic-Vlahovic, M. (2000). A modified 420 microtiter-plate test for quantification of staphylococcal biofilm formation. *Journal of Microbiological Methods, 40*, 175–179.

Trivedi, K., Cupakova, S., & Karpiskova, R. (2011). Virulence factors and antibiotic resistance in enterococci isolated from food-stuffs. *Veterinary Medicine, 56*, 352–357.

Vebø, H. C., Snipen, L., Nes, I. F., & Brede, D. A. (2009). The transcriptome of the nosocomial pathogen *Enterococcus faecalis* V583 reveals adaptive responses to growth in blood. *PLos One, 4*, 1–14.

Vinderola, C. G., & Reinheimer, J. A. (2003). Lactic acid starter and probiotic bacteria: a comparative "*in vitro*" study of probiotic characteristics and biological barrier resistance. *Food Research International, 36*, 895–904.

Yap, I. K. S., Li, J. V., Saric, J., Martin, F. P., Davies, H., Wang, Y., et al. (2008). Metabolomic and microbiological analysis of the dynamic effect of vancomycin-induced gut microbiota modification in mice. *Journal of Proteome Research, 7*, 3718–3728.