Safety, beneficial and technological properties of Enterococcus faecium isolated from Brazilian cheeses

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Abstract

This study aimed to characterize the safety and technological properties of Enterococcus faecium strains isolated from Brazilian Coelho cheeses. High levels of co-aggregation were observed between Enterococcus faecium strains EM485 and EM925 and both Escherichia coli and Clostridium perfringens. Both strains presented low levels of hydrophobicity. E. faecium EM485 and EM925 were both able to grow in the presence of 0.5% of the sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), and glycodeoxycholic acid (GDC), although they showed the ability to deconjugate only GDC and TDC. Both strains showed good survival when exposed to conditions simulating the gastrointestinal tract (GIT). When tested for the presence of virulence genes, only tyrosine decarboxylase and vancomycin B generated positive PCR results.

Key words: Enterococcus faecium, probiotic properties, technological properties, virulence factors, antibiotic resistance.

Introduction

Enterococcus spp. presents very high survival in the presence of salts and variable pH and is adapted to several food systems. In the Mediterranean region, Enterococcus spp. have played important roles in the preparation of various fermented milk and meat products for centuries, and they are essential for the ripening of cheese products and to the development of their aroma (Giraffa, 2002; Foulequié-Moreno et al., 2006; Franz et al., 2011) due to proteolysis, lipolysis, and the production of diacetyl (Giraffa, 2003). In addition, various studies have shown that bacteriocinogenic lactic acid bacteria (LAB), including Enterococcus spp., are commonly isolated from Brazilian dairy products (Gomes et al., 2008; Frazzon et al., 2010; Moraes et al., 2010; Ortolani et al., 2010).

Numerous studies in the last decade have demonstrated the safe application of enterococci in foods (Franz et al., 2011; Ogier and Serror, 2008; Martin-Platero et al., 2009), and certain enterococci have been investigated with regard to their potential as probiotics (Franz et al., 2003; Fouliquié-Moreno et al., 2006; Botes et al., 2008; Todorov and Dicks, 2008). The application of Enterococci as a starter culture or a probiotic has been increasingly investigated, and Enterococcus faecium SF68® (NCIMB 10415, Cerbios-Pharma SA, Barbengo, Switzerland) and E. faecalis Symbioflor 1 (SymbioPharm, Herborn, Ger-
many) have been successfully applied for the treatment of diarrhea in dogs and cats (Bybee et al., 2011). In addition, E. durans M4-5 was found to produce butyrate, which induces significant anti-inflammatory effects and helps preserve the epithelial integrity of the intestine (Raz et al., 2007; Avram-Hananel et al., 2010). Enterococci probiotics are also used to prevent or treat diarrhea in pigs, poultry, cattle, and pets (Franz et al., 2011). However, their role as probiotics is still controversial because of their increased association with nosocomial infections and because they harbor multiple antibiotic-resistant genes, which are transmissible by conjugation to pathogenic microorganisms (Dicks et al., 2011; Franz et al., 2011; Montalban-Lopez et al., 2011). Several putative virulence factors have been described in enterococci, such as aggregation substance protein, gelatinase, cytolsin, enterococcal surface proteins, hyaluronidase, accessory colonization factors and endocarditis antigens (Vankerckhoven et al., 2004; Martin-Platero et al., 2009).

In this study, bacteriocin-producing strains isolated from artisanal Coalho cheese produced in Ceará state, Brazil (dos Santos et al., 2014) were evaluated regarding their beneficial and technological potential. The strains have been identified to be E. faecium (dos Santos et al., 2014). Moreover, their safety and technological properties were determined. To our knowledge, this is the first report on the characterization of beneficial E. faecium strains isolated from Coalho cheese with potential technological applications.

Materials and Methods

Strains

E. faecium EM485 and E. faecium EM925 have been previously isolated from Coalho cheese and identified based on biochemical, physiological and genetic properties (dos Santos et al., 2014). Bacterial cultures were maintained in the presence of 20% glycerol at -80 °C.

Safety evaluation

Antimicrobial susceptibility

Antimicrobial Susceptibility Test Discs (Oxoid, Basingstoke, UK) were employed to assess the susceptibility of selected enterococcus strains to antimicrobials classified as inhibitors of cell envelope synthesis (penicillin G, ampicillin and vancomycin), protein synthesis inhibitors (gentamicin, streptomycin, tetracycline, chloramphenicol, and eritromycin), and inhibitors of nucleic acid synthesis (co-trimoxazole, rifampicin and metronidazole). MRS agar (Difco) plates containing 10^5-10^7 cfu/mL of E. faecium EM485 or E. faecium EM925, respectively, were prepared after strain cultivation in MRS broth at 37 °C for 48 h. Discs impregnated with the antimicrobials were applied to the plates, which were subsequently incubated at 37 °C for 24 h. Inhibition zones around the discs were measured, and the strain was considered resistant to the antimicrobial agent according to the size of the inhibition zone (Charteris et al., 1998). The test was performed in triplicate.

In addition, the MIC (minimum inhibition concentration) was determined by using MICE Test strips (Oxoid, Basingstoke, UK). MRS agar plates containing 10^5-10^7 cfu/mL were prepared after strain cultivation in MRS broth at 37 °C for 48 h. Antibiotic strips impregnated with a gradient of antimicrobials were applied to the plates, which were subsequently incubated at 37 °C for 24 h. Inhibition zones around the strips were recorded. The test was performed in triplicate.

Characterization of virulence potential

E. faecium EM485 and E. faecium EM925 were tested for the following virulence genes: gelE (gelatinase), hyl (hyaluronidase), asa1 (aggregation substance), esp (enterococcal surface protein), cytA (cytolysin), efaA (endocarditis antigen), ace (adhesion of collagen), vanA and vanB (both related to vancomycin resistance), and for genes for amino acid decarboxylases: hdc1 and hdc2 (both related to histidine decarboxylase), tdc (tyrosine decarboxylase), and odc (ornithine decarboxylase) using PCR protocols from Martin-Platero et al. (2009), Rivas et al. (2005) and Vankerckhoven et al. (2004). The amplified products were separated by electrophoresis on 0.8 to 2.0% (w/v) agarose gels in 0.5x TAE buffer. The gels were stained in TAE buffer containing 0.5 μg/mL ethidium bromide (Sigma-Aldrich Co.). The primers are detailed in Table 1.

Hydrophobicity

To evaluate cell surface hydrophobicity, overnight stationary-phase cultures of E. faecium EM485 and E. faecium EM925 were centrifuged at 7000 x g for 5 min at 4 °C (Centrifuge 5810R, Eppendorf, Hamburg, Germany), washed twice with phosphate buffer (50 mM K2HPO4/KH2PO4, pH 6.5), and re-suspended in the same buffer until A560 values (A0) near 1.0 were obtained. N-hexadecane was then added to the cell suspension (1:5) and the mixture was vortexed for 120 s. After a period of 1 h at 37 °C, the A560 value (A) of the aqueous layer was measured. Cell surface hydrophobicity was calculated according to the equation: %H = [(A0 - A)/A0] x 100, where A0 and A are the absorbance values before and after extraction with the organic solvent, respectively. The assay was performed in sextuplicate.

Beneficial and technological properties

Viability in milk acidified with lactic acid

The growth in milk was evaluated as described by Vinderola et al. (2008) with some modifications. E. faecium EM485 and E. faecium EM925 were activated in MRS broth for 16 h at 35 °C. The cultures (1 mL) were centrifuged (6000 x g, 15 min, 4 °C) and washed twice with buffered phosphate saline (PBS) solution, pH 7.4. The cells
were re-suspended to 10 mL with reconstituted skim milk (10%, w/v) and incubated at 35 °C. Changes in milk pH were recorded after 6, 24 and 48 h of incubation.

Each cell suspension (1.5%, v/v) was transferred to skim milk (10%, w/v) previously acidified with lactic acid to pH 4.0 or 5.0. The control was skim milk without added lactic acid. Cultures were incubated up to 30 days at 5 °C. Strains viability was tested by plating on MRS agar and counting colonies on days 0 and 30 as described by Vinderola et al. (2002).

Effect of simulated gastric and intestinal conditions on the viability of selected strains

To compare the survival of *E. faecium* EM485 and *E. faecium* EM925 throughout a simulated gastric and intestinal passage, an *in vitro* model was employed, adapted from Pinto et al. (2006). MRS broth was inoculated with approximately 2 x 10^8 cfu/mL of an overnight culture, and an aliquot of 1 mL was serially diluted in peptone water, poured-plated onto acidified MRS agar (pH 5.4), and incubated anaerobically at 37 °C for 72 h to determine the cfu/mL at time 0. To simulate gastric conditions, 6 mL of the cell suspension was diluted in 10 mL of an artificial gastric fluid consisting of a sterile electrolyte solution (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L CaCl_2 and 1.2 g/L NaHCO_3, pH adjusted to 2.5) with 0.3% pepsin (Sigma-Aldrich, St. Louis, USA) and incubated 1 h at 37 °C under agitation (150 rpm; Dubnoff Bath, Tecnal, Piracicaba, Brazil). After this period, a 1 mL aliquot was removed, ser-

### Table 1 - Primers sequences utilized in the investigation of presence/absence for virulence factors, vancomycin resistance and biogenic amine production.

| Primers (5’ - 3’ ) | Reference |
|--------------------|-----------|
| Virulence genes*     |           |
| gelE                | TATGACAAATGCTTTTGGGAT Vankerckhoven et al., 2004 |
|                     | AGATGCACCCGAAATAATATA |
| Hyl                 | ACAGAAAAGAGCTGCAGGAAGAT Vankerckhoven et al., 2004 |
|                     | GACTGACGTCCAAGTTTCCAA |
| asa1                | GCACGCATATTAGAAATAGG A Vankerckhoven et al., 2004 |
|                     | TAAGAAAGAACATCACCACGA |
| Esp                 | AGATTTCTACCTTTGATCTTG Vankerckhoven et al., 2004 |
|                     | AATTGATCTTTAGCATCTGG |
| cylA                | ACTCGGGGATTGATAGGC Vankerckhoven et al., 2004 |
|                     | GCTGCTAAGTGGCCTTTT |
| efaA                | GCCAATTTGGAGACACGCCCT Martin-Platero et al., 2009 |
|                     | CGCCTTCTGTCTCTTCTTGTG |
| Ace                 | GAATTGAGCAAAGATCCATCG Martin-Platero et al., 2009 |
|                     | GTCTGCTTTTCTACCTGTTTC |
| Antibiotic resistance |          |
| VanA                | TCTGCAATAGAGATAGCCG Martín-Platero et al., 2009 |
|                     | GGAATGAGCTATCCACGATT |
| VanB                | GCTCCGCAGCTGTGATGGCA Martín-Platero et al., 2009 |
|                     | AGATGCCGGCATCTCTCTG |
| Biogenic amine      |            |
| hdc1                | AGATGGATTTTGCTTAGT Rivas et al., 2005 |
|                     | AGACCATACACCAAACCTT |
| hdc2                | AAYTCNTTYGAYTYTGGARAGARG Rivas et al., 2005 |
|                     | ATNGGNGANCDDATCATYTTTRTNCC |
| Tdc                 | GAYATNATNGGATNGNYTNGACARG Rivas et al., 2005 |
|                     | CCRTARTCGNGNATAGCRAARTCNTGRTG |
| odc                 | GTNTTYAAYGCGAYAARCNTAYTYTGYT Rivas et al., 2005 |
|                     | ATNGARTTNGATTTGACTYYYTTCNGG |

Positive results (+) for genes for virulence and biogenic amines in *E. faecium* EM485 and EM925. *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), and *odc* (ornithine decarboxylase).
ally diluted in peptone water, pour-plated onto acidified MRS agar, and incubated anaerobically at 37 °C. To simulate passage through the small intestine, 2 mL of the remaining suspension was diluted in 8 mL of artificial duodenal secretion (pH 7.2) consisting of 6.4 g/L NaHCO₃, 0.239 g/L KCl, 1.28 g/L NaCl, 0.5% bile salts (Oxgall, Merck, Darmstadt, Germany) and 0.1% pancreatin (Sigma-Aldrich Co., St. Louis, USA). After 3 h of incubation at 37 °C under agitation (150 rpm), 1 mL aliquots were removed to determine the final cfu/mL. The assay was performed three times for each strain, and the enterococcus enumeration was performed in triplicate.

### β-galactosidase activity

The β-galactosidase activity of *E. faecium* EM485 and *E. faecium* EM925 was assessed employing sterile filter paper disks impregnated with o-nitrophenyl-β-D-galactopyranose (ONPG Disks, Fluka, Buchs, Switzerland) according to the manufacturer instructions. Overnight cultures of each strain were streaked on MRS agar plates and incubated anaerobically (GasPack System, Oxoid, Basingstoke, Hampshire, UK) at 37 °C for 48 h. A colony of each strain was picked up and emulsified in a tube containing an ONPG disk with 0.1 mL of sterile 0.85% (w/v) sodium chloride solution. The tubes were incubated at 35 °C and observed at intervals of one hour for up to 6 h. The release of a yellow chromogenic compound, o-nitrophenol, indicates a positive colony. The test was performed twice for each strain, in duplicate.

### Bile salt deconjugation

To evaluate the ability of *E. faecium* EM485 and *E. faecium* EM925 to perform bile salt deconjugation, overnight cultures of each isolate were streaked on MRS agar plates and incubated anaerobically (GasPack System, Oxoid, Basingstoke, Hampshire, UK) at 37 °C for 48 h. A colony of each strain was picked up and emulsified in a tube containing an ONPG disk with 0.1 mL of sterile 0.85% (w/v) sodium chloride solution. The tubes were incubated at 35 °C and observed at intervals of one hour for up to 6 h. The release of a yellow chromogenic compound, o-nitrophenol, indicates a positive colony. The test was performed twice for each strain, in duplicate.

### Proteolytic activity

*E. faecium* EM485 and *E. faecium* EM925 were cultured in 5 mL of MRS broth for 24 h at 37 °C. The cells were harvested by centrifugation at 10 000 x g for 30 min at 4 °C. The pellets were washed three times with 0.9% (w/v) NaCl solution, resuspended in 10 mL of sterile 10% (w/v) skim milk, and incubated for 24 h at 37 °C. A control group with 10 mL of sterile 10% skim milk, but without inoculum, was incubated under the same conditions. At the end of the incubation the fermented and control groups were centrifuged at 10 000 x g for 30 min at 4 °C. The supernatant was designated as the extracellular enzymatic extract. After withdrawal of the extracellular enzymatic extract, the obtained pellets were washed with 25 mM Tris-HCl, pH 7.5 and submitted to a protocol of Tsakalidou et al. (1999) to prepare a cell wall extract. In all steps, the protein content of the extracts was estimated using the method of Bradford (1976). Bovine serum albumin was used as a standard.

The protease assay was carried out as described previously by Church et al. (1983) using α-phthaldialdehyde (OPA). The proteolytic activity of the stains in fermented milk was expressed as the absorbance of free amino groups measured at 340 nm, and specific activity was calculated by dividing the protease activity values by the protein content results. One unit of protease activity was defined as the amount of enzyme required to produce an increase of 0.001 in the optical density at 340 nm of the fermented milk relative to that of the unfermented milk (milk blank).

To investigate the locations of the proteolytic enzymes, 100 µg of protein from either the extracellular enzymatic extract or the cell wall extract was assayed by zymogram. Sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (10.0% polyacrylamide gel) according to Laemmle (1970) with 0.1% gelatin included in the gel was applied. To re-nature the enzymes after SDS-PAGE, the gels were treated twice with Triton-X 100 for 30 min each time, washed with water and incubated with 20 mM CaCl₂ in 25 mM Tris-HCl, pH 7.5 at 37 °C for 72 h. The gels were stained with silver. All the analyses were carried out in triplicate. Multi-comparison of means was assessed by Student *t*-test at the 0.05 (p < 0.05) level of statistical significance.

### Aggregation

Aggregation of *E. faecium* EM485 and *E. faecium* EM925 with *Escherichia coli* INCQS 00033 and *Clostridium perfringens* INCQS 00130.

To evaluate auto-aggregation, strains *E. faecium* EM485 and *E. faecium* EM925 were grown in MRS broth for 24 h at 37 °C. The cells were harvested by centrifugation at 7000 x g for 10 min at 20 °C, washed, resuspended and diluted in 0.85% sterile saline to OD₆₀₀nm = 0.3. One milliliter of the cell suspension was transferred to a 2 mL sterile plastic cuvette and the OD₆₆₀nm was recorded over 60 min using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech). Auto-aggregation was determined using the following equation (Todorov and Dicks, 2008):

\[
\text{% Auto-aggregation} = \left( \frac{[\text{OD}_0 - \text{OD}_{60}]}{\text{OD}_0} \right) \times 100
\]

OD₀ refers to the initial OD and OD₆₀ refers to the OD determined after 60 min. For determination of OD₆₀ the cultures were centrifuged at 300 x g for 2 min at 20 °C.

To evaluate co-aggregation, strains *E. faecium* EM485 and *E. faecium* EM925 were grown in 10 mL MRS and *Escherichia coli* INCQS 00033 and *Clostridium* (C.) *perfringens* INCQS 00130 were grown in BHI or MRS at
37 °C. Cells were harvested after 24 h (7000 x g, 10 min, 20 °C), washed, re-suspended and diluted in 0.85% sterile saline to OD_{660nm} = 0.3. Then, 500 µL of each suspension was mixed in a 2 mL sterile plastic cuvette and the OD_{660nm} was recorded over 60 min using a spectrophotometer (Ultraspec 2000, Pharmacia Biotech). The degree of co-aggregation was determined by OD readings of mixed cultures. Co-aggregation was calculated using the following equation (Todorov and Dicks, 2008):

\[
% \text{ Co-aggregation} = \left[ \frac{(OD_{\text{tot}} - OD_{s})}{OD_{\text{tot}}} \right] \times 100
\]

OD_{tot} refers to the initial OD, taken immediately after the tested strains were mixed, and OD_{s} refers to the OD of the supernatant after 60 min. The experiments were conducted in triplicate on two separate occasions.

**Results and Discussion**

**Safety evaluation**

*Effect of antibiotics on the growth of E. faecium EM485 and E. faecium EM925*

According to the results of the disc diffusion method (data not shown), the two studied *E. faecium* strains, EM485 and EM925, demonstrated susceptibility to penicillin G, ampicillin, chloramphenicol, tetracycline, erythromycin, and rifampicin. On the other hand, both strains showed high-level resistance to ciprofloxacin, vancomycin, and metronidazole (MIC values > 256.0 µg/mL), and also presented a MIC value for tetracycline (4.0 µg/mL) higher than the MIC breakpoint defined by EFSA (2008) for this antibiotic (Table 2). Resistance to streptomycin, co-trimoxazole (the MICs for these two antibiotics were not determined in this study) and metronidazole were also detected for both strains with the disc diffusion method. In relation to gentamicin, although the two strains showed resistance in the disc diffusion method (inhibition zone < 12 mm), and the MIC values were 16.0 and 32.0 µg/mL for *E. faecium* EM485 and EM925, respectively, not exceeding the MIC breakpoint established for *Enterococcus* spp. (EFSA 2008). Both strains presented MIC values of 4.0 µg/mL for oxacillin and 8.0 µg/mL for levofloxacin. The resistance of *E. faecium* EM485 and *E. faecium* EM925 to co-trimoxazole and metronidazole could be an acquired feature considering the resistance to antibiotics commonly reported for *Enterococcus* spp. in the literature, and it deserves further investigation. Favaro et al. (2014) reported that four *E. faecium* strains isolated from feta cheese (from Bulgaria) were susceptible to ampicillin and penicillin and 2 of them were susceptible to vancomycin, which are the most clinically relevant antibiotics.

| Antibiotic | MIC breakpoint recommendation of EUCAST (2011) and EFSA (2008) for *Enterococcus* spp. | Enterococcus faecium EM485 | Enterococcus faecium EM925 | Sensitivity or resistance |
|------------|----------------------------------------------------------------------------------------|----------------------------|----------------------------|--------------------------|
| Amoxicillin | S ≤ 4 µg/mL, R ≥ 8 µg/mL | 0.12 µg/mL | 0.06 µg/mL | S |
| Amoxicillin / clavulonic acid | S ≤ 4 µg/mL, R ≥ 8 µg/mL | 0.12 µg/mL | 0.25 µg/mL | S |
| Ampicillin | S ≤ 4 µg/mL, R ≥ 8 µg/mL | 0.5 µg/mL | 0.12 µg/mL | S |
| Cefotaxime | NS | 0.25 µg/mL | 0.25 µg/mL | S |
| Ceftriaxone | NS | 0.12 µg/mL | 0.06 µg/mL | S |
| Ciprofloxacin | NS | - | - | R |
| Erythromycin | NS | 0.25 µg/mL | 0.5 µg/mL | S |
| Gentamicin | > 128 µg/mL | 16.0 µg/mL | 32.0 µg/mL | S |
| Imipenem | S ≤ 4 µg/mL, R ≥ 8 µg/mL | 0.015 µg/mL | 0.015 µg/mL | S |
| Levofloxacin | NS | 8.0 µg/mL | 8.0 µg/mL | S |
| Linezolid | NS | 1.0 µg/mL | 1.0 µg/mL | S |
| Meropenem | NS | 0.015 µg/mL | 0.015 µg/mL | S |
| Metronidazole | NS | - | - | R |
| Oxacillin | NS | 4.0 µg/mL | 4.0 µg/mL | S |
| Penicillin | NS | 1.0 µg/mL | 1.0 µg/mL | S |
| Tetracycline | NS | R > 2 µg/mL | 4.0 µg/mL | R |
| Vancomycin | S ≤ 4 µg/mL, R > 4 µg/mL | - | R |

- = growth of tested *Enterococcus faecium* strain was not effected by antibiotic; * with potential interference form the medium; NS = not specified by cited documents; R = resistant; S = sensitive.
for curing infections involving multiple antibiotic-resistant enterococcus strains. In a study of Schirru et al. (2012), the majority of the tested antibiotics inhibited the growth of four *E. faecium* strains isolated from goat's milk to some extent (from Sardenia, Italy). In the same study, five of the tested antibiotics (cotrimazin, cotrimixazol, nalidixic acid, oxacillin and sulphonamid) had no inhibitory effect on the studied *E. faecium* strains. In addition, variable results have been reported for interactions between *E. faecium* strains and cefalotin, cepfime, ceftriafor oflaxacim, tazobactam, amoxicillin + clavulanic acid and ampicillin + sulbactam (Schirru et al., 2012).

Antibiotic resistance is a fundamental issue in the safety evaluation of *Enterococcus* spp. for use in food products. Acquired resistance located in plasmids and transposons is the main concern (Giraffa, 2002; Franz et al., 2005). Moreover, intrinsic resistance to some antibiotics is a feature commonly found among *Enterococcus* strains isolated from foods, as reported by Franz et al. (2005).

Antibiotic resistance in genus *Enterococcus* is controversial. Enterococci have a long history of application in food production (Schillinger et al., 1996) due to their favorable metabolic activities (lipolysis, esterolysis, citrate utilization, etc.) that contribute to the typical taste and flavor of fermented foods (Centeno et al., 1996; Giraffa and Carminati, 1997; Manolopoulou et al., 2003). However, some *Enterococcus* strains are pathogens for humans and other animals, and vancomycin-resistant *Enterococcus* spp. are frequently resistant to many antibiotics commonly used in veterinary and human medicine (Landman and Quale, 1997).

In the application of enterococci in foods, we need to pay special attention because of the possible spread of genetic determinants of resistance from these bacteria, which are generally located in conjugative plasmids or transposons prone to genetic exchange (Hasman et al., 2005; Zanella et al., 2006). Multi-resistance has been more commonly reported for *E. faecalis* due to its notorious ability to acquire and transfer antibiotic-resistance genes (Citak et al., 2004; McBride et al., 2007). Moreover, Gomes et al. (2008) reported that the prevalence of antibiotic resistance was higher for *E. faecalis* compared to *E. faecium* isolates. The results presented in the literature indicate that foods cannot be ruled out as a potential source for spreading antibiotic-resistant strains. (Abriouel et al., 2008). Tomé et al. (2008) reported resistance to chloramphenicol by a strain of *E. faecium* isolated from cold-smoked salmon evaluated for its potential application as biopreservative. However, it is important to consider that starter cultures and potential probiotic LAB may be a potential reservoir of antibiotic resistance genes and that horizontal gene transfer to the other bacteria present in the human GIT is a possible scenario (Dicks et al., 2011; Teuber, 1999; Salyers et al., 2004).

**Genes for virulence, biogenic amines and antibiotic resistance**

Verification of virulence factors in *Enterococcus* spp. by bio-molecular and bio-chemical approaches is important due to the risk of genetic transfer because these genes are usually located in conjugative plasmids (Eaton and Gasson, 2001). In tests of the genes adhesion of collagen protein, aggregation substance, cytolysin, endocarditis antigen, enterococcal surface protein, gelatinase, hyaluronidase, histidine decarboxylase, ornithine decarboxylase, tyrosine decarboxylase, vancomycin A and vancomycin B, the only positive results were generated by PCR targeting the tyrosine decarboxylase and vancomycin B genes in *E. faecium* EM485 and *E. faecium* EM925 (Table 1). In general, the observed frequency of positive results for the virulence factors studied was lower than that reported in other studies on *Enterococcus* isolated from foods (Gomes et al., 2008; Barbosa et al., 2010) and lower when compared to studies with clinical isolates (Eaton and Gasson, 2001; Franz et al., 2001; Barbosa et al., 2010).

The investigation of virulence factors in *Enterococci* strains with potential application in food preservation is of foremost importance because such strains may contain several determinants of pathogenicity. Virulence factors may act as colonization factors by promoting the adhesion of bacteria to host cells, or as invasion factors that promote the invasion of epithelial cells, which disrupts the immune system (de Souza, 2003). Several cell wall surface proteins play roles in enterococcal pathogenicity, including aggregation substance, enterococcal surface protein, and collagen-binding components (Hendrickx et al., 2009). Some extracellular proteins, such as hyaluronidase, may interact with lymphocyte receptors and be responsible for the induction of auto-immune diseases (de Souza, 2003). Cytolysin is an exotoxin with bifunctional function as a bacteriocin and by presenting hemolytic effects (Haas et al., 2002). Expression of the aggregation substance protein facilitates close contact between cells, conjugation and possible transfer of virulence plasmids (Hendrickx et al., 2009). The aggregation substance protein may have a role in the translocation of enterococci into epithelial cells (Franz and Holzapfel, 2004). *Enterococcus* surface protein is a cell wall-anchored protein with a special role in biofilm formation (Hendrickx et al., 2009). ACE (angiotensin converting enzyme) proteins facilitate the binding of *Enterococcus* spp. to collagen and are involved in pathogenicity in human infections (Franz and Holzapfel, 2004). Gelatinase production is usually associated with enterococci from clinical samples, but it has also been detected in enterococci isolated from dairy and meat products (Archimbaud et al., 2002). The role of hyaluronidase in infections has been reviewed by Girish and Kemparaju (2007). Hyaluronidase facilitates the spread of bacteria and toxins throughout host tissue by causing tissue damage (Kayaoglu and Orstavik, 2004). In enterococci, six vanco-
mycin resistance types have been phenotypically and geno-
typically identified, and two of them, VanA and VanB, may
be located in transferable plasmids (Courvalin, 2006).

**Hydrophobicity**

Several mechanisms are involved in the adhesion of microorganisms to intestinal epithelial cells. The hydro-
phobic nature of the outermost surface of microorganisms
is involved in the attachment of bacteria to host tissue. The
determination of microbial adhesion to hexadecane as a
way to estimate the ability of a strain to adhere to epithelial
cells is a valid qualitative phenomenological approach
(Kiely and Olson, 2000).

Both strains presented low levels of hydrophobicity
(8.18% for *E. faecium* EM485 and 11.33% for *E. faecium*
EM925) determined as adhesion to n-hexadecane. Cell sur-
face hydrophobicity is a non-specific interaction between
microbial cells and their host. The initial interaction may be
weak, is often reversible and it precedes subsequent adhe-
sion processes mediated by more specific mechanisms
involving cell-surface proteins and lipoteichoic acids (Gra-
nato *et al.*, 1999; Rojas *et al.*, 2002; Ross and Jonsson,
2002). Bacterial cells with high hydrophobicity usually
present strong interactions with mucosal cells. The same
range of hydrophobicity has been reported for *E. faecium*
isolated from feta cheese (from Bulgaria), presenting levels
of hydrophobicity between 7.92% and 10.23% (Favaro 
*et al.*, 2014). In another study (Todorov *et al.*, 2011), hydro-
phobicity values between 12.6% and 14.7% were recorded
for *E. faecium* ET05, ET12 and ET88. These values are
lower than that recorded for *L. rhamnosus* GG (53.3%,
Todorov *et al.*, 2008). Hydrophobicity may assist in adhe-
sion, but it is not a prerequisite for strong adherence.
Hydrophobicity varies among genetically closely related
species and even among strains of the same species
(Schar-Zammarretti and Ubbink, 2003).

**Beneficial and technological properties**

**Growth in milk and viability in milk acidified with lactic acid**

*E. faecium* EM485 and EM925 both grew in milk and
were able to change milk’s pH after 6, 24 and 48 h of incu-
bation. No decrease in cell counts was observed when
*E. faecium* EM485 and *E. faecium* EM925 were maintained
in milk acidified to pH 4.0 or to pH 5.0 at 5 °C for 30 days
(Table 3). These technological characteristics are interest-
ing for posterior use in acidic products such as yogurts and
acidified milk. The acidifying activity of *E. faecium* iso-
lated from goat’s milk (from Sardenia, Italy) reported by
Schirru *et al.* (2012) was generally low. A good acid-
producing starter culture needs to reduce the pH of milk
from its normal value of approximately 6.6 to 5.3 in 6 h us-
ing an inoculum of 10%, and in general, enterococci exhibit
low milk acidifying ability (Schirru *et al.*, 2012).

**Effect of simulated gastric and intestinal conditions on the
viability of selected strains**

When *E. faecium* EM485 and *E. faecium* EM925 was
exposed to simulated gastric and small intestine conditions,
good survival rates were recorded for both strains (Figure
1). Such results are not surprising because *Enterococcus*
spp. have a good chance of surviving acidic and bilious
conditions (Todorov *et al.*, 2011). A cell count reduction
of 3.98 log cfu/mL was recorded for *E. faecium* EM485 after
exposure to simulated gastric and small intestine condi-
tions. Similar results were recorded for *E. faecium* EM925,
with a reduction in cell number of 3.03 log cfu/mL (Fig-
ure 1).

Probiotics must survive in the acidic gastric environ-
ment if they are to reach the small intestine and colonize the
host, thereby imparting their benefits. *Enterococcus*
species are considered intrinsically resistant to acid (Tannock,
2004). Although there are differences between species and
strains, organisms generally exhibit increased sensitivity
at pH values below 3.0 (Ronka *et al.*, 2003). Hence, acid
tolerance is accepted as one of the desirable properties used
to select potentially probiotic strains. Gastric transit studies
of probiotics have been conducted using both simulated
gastric juice and animal and human gastric juices (Charteris
*et al.*, 1998; Gardiner *et al.*, 1999). Both of these ap-
proaches have limitations; the former fails to capture the in-
fluence of dietary and nonacidic constituents of gastric
secretions on probiotic survival, and the latter is restricted
by the availability of fresh material (Charteris *et al.*, 1998).
In addition, the exploitation of rich media, such as acidified
MRS medium, may offer protection to bacteria by provid-
ing energy and metabolic precursors. The use of food ingre-
dients to enhance probiotic survival through the GIT has
been extensively studied (Charteris *et al.*, 1998; Gardiner
*et al.*, 1999). However, little data are available describing the
effects of individual food components and the underlying

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**Table 3 - Growth in milk and viabiliy in milk acidified with lactic acid.**

| Microorganism | Milk’s pH after | Viability (Δ log cfu/mL) in acidified milk at |
|---------------|----------------|------------------------------------------|
|               | 6 h  | 24 h | 48 h | pH 4.0 | pH 5.0 |
| *E. faecium* EM485 | 5.8  | 5.2  | 4.8  | 0.6a  | 0.95b |
| *E. faecium* EM925 | 5.3  | 4.8  | 4.8  | 0  | 0.47b |

aDifference between counts at time 0 and after 30 days of cold storage (5 °C) in milk acidified at different pH values.
bIncrease of viable cell counts.
mechanisms of action whereby they enhance the survival of LAB (Charalampopoulus et al., 2003).

Production of β- galactosidase

The ability of microorganisms to ferment lactose in milk is an important technological property of LAB with potential applications in the dairy industry. Hydrolysis of lactose, which confers taste, texture and nutritional value to milk and milk derivatives is carried out by the enzymes β-D-galactosidase (EC 3.2.1.23) and/or phospho-β-D-galactosidase (EC 3.2.1.85), and it has been described in various organisms such as bacteria, yeasts and molds (Zárate and Chaia, 2012). In addition to their technological importance, both the pure enzymes and the viable microorganisms that contain them have been used to alleviate intestinal disorders such as lactose intolerance. This condition occurs worldwide among the adult population and has been treated successfully by the incorporation of microorganisms, mainly lactobacilli and/or bifidobacteria, into dairy products as a source of β-galactosidase for the intra-intestinal hydrolysis of lactose and the modulation of colonic microbiota (Zárate and Chaia, 2012). In this sense, β-galactosidase activity is a beneficial characteristic for probiotics or LAB with application in the dairy industry.

The production of β-galactosidase by E. faecium EM485 and E. faecium EM925 was confirmed by employing sterile filter paper disks impregnated with o-nitrophenyl-β-D-galactopyranose (ONPG Disks, Fluka, Buchs, Switzerland). In previous studies, β-galactosidase production has been reported in different strains of Enterococcus spp. (Todorov et al., 2010; Favaro et al., 2014).

Deconjugation of bile salts

In our study we found that E. faecium EM485 and E. faecium EM925 were similar in their ability to deconjugate bile salts. Both strains grow on MRS agar plates containing 0.5% (w/v) sodium salts of taurocholic acid (TC), taurodeoxycholic acid (TDC), glycocholic acid (GC), or glycodeoxycholic acid (GDC). However, deconjugation was recorded only for TDC and GDC.

The major route of cholesterol excretion from humans and other mammals is through feces. Cholesterol is the precursor of primary bile salts that are formed in the liver and are stored as conjugated bile salts in the gall bladder for secretion in the gastrointestinal tract (Corzo and Gilliland, 1999). A small fraction of bile salts that is not absorbed is lost as free bile salts in feces. Free bile salts are less soluble than conjugated bile salts, resulting in lower absorption in the intestinal lumen (Center, 1993). At the physiological pH of the intestinal lumen, deconjugated bile salts can be transported through the epithelium (Wong et al., 1994) and into the blood stream of the host, or they can precipitate. Thus, in a steady-state situation, deconjugation of bile acids can reduce serum cholesterol levels by increasing the formation of new bile acids that are needed to replace those that have escaped the enterohepatic circulation (Reynier et al., 1981). Experiments with germ-free rats have shown that bile salt deconjugation by B. longum increases bile salt excretion (Chikai et al., 1987).

Proteolytic activity

The reduction in protein concentration in extracellular enzymatic extract by E. faecium EM485 and E. faecium EM925 grown in milk for 24 h in comparison with the control (Figure 2) shows that the two strains display proteolytic activity. However, this enzymatic activity was very low when compared to that reported by Donkor et al. (2007) for Lb. casei L26, which presented OD_{340 nm} above 1.80 after 24 h of fermentation in MRS at 42 °C. Church et al. (1983) observed OD_{340 nm} above of 0.40 after Streptococcus (St.) lactis C2 fermentation in milk for 24 h at 22 °C.

The specific proteolytic activity of E. faecium EM485 and E. faecium EM925, calculated from the proteolytic ac-
activity and protein concentration values, were 18.73 ± 3.90 U/mg protein and 24.55 ± 2.57 U/mg protein, respectively, without a significance difference between them (p > 0.05, t-test). As shown in Figure 2, the enzymatic activity of both strains is located in the extracellular extract. This finding is consistent with Arizcum et al. (1997) reporting that the enzymatic activity of Enterococcus spp. was located mainly in the extracellular extract fraction.

The importance of the protease activity is that it is related to the good growth of LAB in milk and to casein hydrolysis during cheese ripening. A study of Schirru et al. (2012) reported low proteolytic activity in 2 of 4 tested E. faecium strains isolated from goat’s milk (from Sardenia, Italy). However, even though lower values have been reported for other Enterococcus spp. strains (Pepe et al., 2003), the result was similar to those produced by several LAB strains described in Madrall et al. (2006) as promising autochthonous starter cultures for the production of Pecorino sardo cheese.

Aggregation

Auto-aggregation proved to be strain-specific and may vary inside the same taxonomic group. The value re-

Figure 2 - Proteolytic activity (A) Extracellular proteolytic activity measured from two Enterococcus faecium strains compared with that of unfermented milk (milk blank). The strains were grown in milk for 24 h at 37 °C and the samples were assayed using the o-phthaldialdehyde method in a spectrophotometer at 340 nm. The data are reported as the mean ± S.E.M (n = 3). Different letters show significant difference (p < 0.05, t-test). (B) Protein concentration in extracellular enzymatic extract of two Enterococcus faecium strains compared with that of unfermented milk (milk blank). The strains were grown in milk for 24 h at 37 °C. The data are reported as the mean ± S.E.M (n = 3). Different letters show significant difference (p < 0.05, t-test).
corded for *E. faecium* EM485 was 80 ± 2% and for *E. faecium* EM925 it was 78 ± 3% (Figure 3). Favaro et al. (2014) reported much lower levels of auto-aggregation for *E. faecium* isolated from feta cheese (from Bulgaria) ranging from 8 ± 1 to 10 ± 2. In another study conducted by Todorov et al. (2008), strain-specificity in auto-aggregation was also observed for *L. pentosus* ST712BZ and *L. paracasei* ST284BZ. High levels of co-aggregation were observed between *E. faecium* EM485 and both *Escherichia coli* INCQS 00033 (78 ± 2%) and *C. perfringens* INCQS 00130 (81 ± 2%) and also between *E. faecium* EM925 and both *Escherichia coli* INCQS 00033 (74 ± 4%) and *C. perfringens* INCQS 00130 (84 ± 3%) (Figure 3). Different degrees of co-aggregation have been measured with *L. ivanovii* subsp. *ivanovii* ATCC 19119, *L. innocua* ATCC 33090, *L. monocytogenes* ATCC 7644 and *E. faecium* strains as reported by Favaro et al. (2014). High auto-aggregation would facilitate the exclusion of these pathogenic species from the GIT. Low levels of co-aggregation with pathogens may play an important role in preventing the formation of biofilms, in this way eliminating the pathogen from the GIT. On the other hand, higher co-aggregation levels with pathogens will facilitate antibacterial action by *E. faecium* EM485 and *E. faecium* EM925 and facilitate pathogen exclusion from the human GIT.

Conclusions

To the best of our knowledge, this is the first study reporting on the properties of *E. faecium* strains isolated from artisanal Coalho cheeses from Brazil that addresses their technological potential, beneficial properties, and safety with respect to the presence of genes encoding virulence factors, biogenic amines and antibiotic resistance. Considering the results, *E. faecium* strains EM485 and EM925 are safe regarding the presence of genes associated with virulence factors and biogenic amine production, and they present technological properties compatible with use in a mixed starter culture for dairy products. However, the resistance to antimicrobials that was detected deserves further investigation.

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Figure 3 - Aggregation properties (auto-aggregation and co-aggregation) for *E. faecium* EM485 and *E. faecium* EM925 with *Escherichia coli* INCQS 00033 and *C. perfringens* INCQS 00130.
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