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# Antimicrobial and antioxidant activities of *Enterococcus* species isolated from meat and dairy products

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## Abstract

Lactic acid bacteria (LAB) have an important role in a great variety of fermented foods. In addition to their contribution to sensory characteristics, they enhance food preservation and can be used as probiotics. In this study, the antimicrobial and antioxidant activities of culture supernatants and cell free extracts of 16 LAB isolated from meat and dairy products were investigated. The bacterial were identified by 16S rRNA sequencing. GenBank BLAST analysis revealed that all the isolates belong to *Enterococcus faecium* species. Antimicrobial activity against the indicator microorganism (*Listeria monocytogenes*) was observed at 11 culture supernatants and 4 cell free extracts. The sensibility of culture supernatant was evaluated by proteinase K and trypsin and it was observed that activity of antimicrobial substance was completely lost after the treatment. All of the isolates showed antioxidant activity as determined by the Thiobarbituric Acid Reactive Substances (TBARS) method with both types of extracts. When the antioxidant capacity was investigated using $ABTS^{\bullet+}$ method (2,2 azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) and DPPH method (2,2-diphenyl-1-picrylhydrazyl) it was observed that only culture supernatants showed antioxidant capacity. These bacteria could particularly help to reduce or inhibit pathogenic microorganisms as well as oxidative spoilage in foods and feed.

*Keywords:* antimicrobial activity, antioxidant, *Enterococcus*, lactic acid bacteria, molecular characterization.

# Atividade antimicrobiana e antioxidante de espécies de *Enterococcus* isoladas de carne e produtos lácteos

## Resumo

As bactérias ácido láticas (BAL) têm um papel importante em uma grande variedade de alimentos fermentados. Em adição à sua contribuição para as características sensoriais, estes microorganismos melhoram a conservação de alimentos e podem ser utilizados como probióticos. Neste estudo, as atividades antimicrobiana e antioxidante do sobrenadante e dos extratos livres de células de 16 isolados de LAB de carne e produtos lácteos foram investigadas. Os isolados foram identificados pelo sequenciamento da região 16S do rRNA. Após a comparação das sequências obtidas com aquelas disponíveis na base de dados GenBank, observou-e que todos os isolados foram pertencentes à espécie *Enterococcus faecium*. A atividade antimicrobiana contra o microrganismo indicador (*Listeria monocytogenes*) foi observada no sobrenadante das culturas em 11 isolados, e nos extratos livres de células por 4 isolados. A sensibilidade da cultura sobrenadante foi avaliada pela proteinase K e tripsina e observou-se que a atividade da substância antimicrobiana foi completamente perdida após o tratamento com as enzimas proteolíticas. Todos os isolados apresentaram atividade antioxidante, como determinado pelo método do ácido tiobarbitúrico de substâncias reativas (TBARS) com ambos os tipos de extratos. Quando a capacidade antioxidante foi investigada usando o método do ABTS (2,2 azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)) e o método de DPPH (2,2-diphenyl-1-picrylhydrazyl) observou-se que apenas os sobrenadantes das culturas demonstraram capacidade antioxidante. Estas bactérias poderiam particularmente ajudar a reduzir ou inibir microorganismos patogênicos, bem como a deterioração oxidativa em alimentos e rações.

*Palavras-chave:* atividade antimicrobiana, antioxidante, *Enterococcus*, bactérias ácido láticas, caracterização molecular.

## 1. Introduction

Lactic acid bacteria (LAB), which include the genera *Lactococcus*, *Streptococcus*, *Lactobacillus*, *Pediococcus*, *Leuconostoc*, *Enterococcus*, *Carnobacterium* and *Propionibacterium*, have an essential role in food fermentations (Chao et al., 2008). Besides the long history of consumption which proves the safety of consuming LAB, lactic cultures have been reported to have health-promoting characteristics that make these microorganisms desirable for use in the production of dairy and other food products (Deraz et al., 2011). The most important contribution of these microorganisms to the product is to preserve the nutritive qualities of the raw material through an extended shelf life and the inhibition of spoilage and pathogenic bacteria. This is due to competition for nutrients and the presence of inhibitors produced by the starter culture, including organic acids, hydrogen peroxide and bacteriocins (O'Sullivan et al., 2002). The ability to produce large quantities of organic acids (mainly lactic acid) through fermentation of food carbohydrates, and consequent pH decrease, are the fundamental factors of the antimicrobial activity of LAB. Organic acids, other inhibitory and beneficial substances, such as hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde, and bacteriocins produced by LAB or substances added to food, act in performance producing a broad spectrum of action against pathogenic and spoilage microorganisms (Cheng and Hoover, 2003; Monteagudo-Mera et al., 2012).

Biological systems provide favorable conditions for occurrence of oxidative reactions that are due to the existence of unsaturated lipids in the cell membranes, and due to abundance of oxidative reactions that occur during regular metabolism. The susceptibility of a cell or a tissue to oxidative stress depends on a number of factors including the availability of antioxidants and the ability for inactivation or elimination of formed oxidized products (Storz and Imlay, 1999; Lobo et al., 2010). Free radicals and other reactive oxygen species (ROS) are generated by exogenous chemicals or endogenous metabolic processes in food systems or the human body. The radicals may cause oxidative damage by oxidizing biomolecules leading to tissue damage and cell death. Atherosclerosis, cancer, emphysema, cirrhosis, and arthritis have been correlated with oxidative damage (Storz and Imlay, 1999; Kapila et al., 2006; Halliwell, 2006; Lobo et al., 2010). Therefore, oxidative damage plays a significant pathological role in human disease. However, ingestion of antioxidative supplements, or foods containing antioxidants, may reduce the oxidative damage on the human body (Wang et al., 2006).

Although, the antioxidative properties of LAB are not studied in detail, it has been shown that numerous LAB species contain NADH oxidase/peroxidase and/or catalase to prevent deleterious oxidative effects (Noonpakdee et al., 2004). The antioxidant effect of LAB in rats showing vitamin E deficiency was studied by Kaizu et al. (1993). The authors demonstrated that some *Lactobacillus* species possess antioxidant activity. Also, they are able to decrease the risk of ROS accumulation during food ingestion.

The antioxidant activity of some species of LAB has been demonstrated by *in vitro* enzymatic assays (Lin and Yen, 1999). The capacity to accumulate selenite ($Se^{4+}$), an essential element showing antioxidant properties (Thirunavukkarasu et al., 2004) has been described for some isolates of *Enterococcus* (Pieniz et al., 2011) and other LAB (Svoboda et al., 2009).

Due the overwhelming importance of LAB in foods and feed, we evaluated the antioxidant capacity and antimicrobial activity of food isolates of *Enterococcus* spp. characterized by molecular analysis of the 16S rRNA gene sequence.

## 2. Material and Methods

### *2.1. Microorganisms*

Thirty six LAB isolated from different meat and dairy products, from the collection culture of the Laboratory of Applied Microbiology and Biochemistry – ICTA, Universidade Federal do Rio Grande do Sul (Porto Alegre, Brazil), were used for selection. Many *Enterococcus* species were isolated from these foods (*E. faecium*, *E. faecalis*, *E. hirae*), and only *E. faecium* species were selected for this study. Among the sixteen isolates used in this work, isolates 2, 3, 5, 6, 7, 8, 9, 12, 17, 20, 24 and 32 were isolated from Minas Frescal (typical Brazilian soft cheese) and B22, C5, IS196 and IS197 from regional homemade sausage. Isolates were kept as frozen stock cultures in Brain Heart Infusion (BHI, Difco Laboratories, Detroit, MI, USA) broth containing 20% (v/v) glycerol. Bacteria were grown in BHI broth at 37°C and thereafter inoculated to plates of BHI with 1.5% agar and incubated at 37°C for 24h. The indicator organism tested in this study was *Listeria monocytogenes* ATCC 7644.

### *2.2. DNA amplification and sequencing*

Isolates were identified by a V3 region of the 16S ribosomal RNA sequencing as follows. The isolates were grown on Brain Heart Infusion agar at 37°C for 24h for evaluation of culture purity. Cells were recovered by centrifugation. DNA was extracted from the cells using Promega Wizard Genomic DNA Purification at Kit (Promega, Madison, WI, USA) with slight modification. Briefly, cells were re-suspended in 300µL of nucleic acid lysis solution, incubated at 80°C for 15min and allowed to cool to room temperature. RNAse solution (1.5µL) was added and incubated at 37°C for 20min. Protein precipitation solution (100µL) was added and the tubes incubated on ice for 5min. Following centrifugation, the supernatant was transferred to a tube and 900 µL ice-cold 95% ethanol was added. The precipitate was recovered by centrifugation. The pellet was washed with 70% room-temperature ethanol and re-suspended in sterile distilled water. Oligonucleotide primers corresponding to positions 27F (5'-AGATTTGATCMTGGCTCAG-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3') were used for PCR amplification of the 16S ribosomal RNA gene (Lane, 1991). The PCR reaction mixture consisted of 12.5µL of PCR master mix (Promega, Madison, WI), genomic DNA template (0.5µL), primer 27F (2.5µL=12.5pmol),

primer 1492R (2.5µL=12.5 pmol) and made up to 25 µL final volume with nuclease-free water. The V3 region of the 16S rRNA gene was amplified using a 35-cycle PCR (initial denaturation, 95°C for 5min; subsequent denaturation, 95°C for 0.5min; annealing temperature, 50°C for 1min; extension temperature, 72°C for 1 min and final extension, 72°C for 5min). The PCR amplification products were analyzed by electrophoresis on a 1% agarose gel. Millipore Montage PCR filter units (Millipore, Billerica, MA) were used to remove primers salts, and unincorporated dNTPs according to the manufacturer's instructions except that an additional 400µL of sterile nuclease free water was added to further remove remaining ingredients of PCR. DNA cycle sequencing was performed using BigDye terminator kit (Applied Biosystems, Foster City, CA) with sequencing primer 519r (5'-GWATTACCGCGGCKGCTG-3') in independent reactions (Institute of Integrative Genome Biology, UCR, CA).

*2.3. Phylogenetic analysis*

GenBank database was used for homology comparisons between the obtained sequences and the ones present in the database. The comparison was performed by using Basic Local Alignment Search Tool (BLAST) available online at www.ncbi.nlm.nih.gov/BLAST/. Phylogenetic and molecular evolutionary analyses were conducted using RDP release 10 software (Cole et al., 2009) and MEGA version 4.1 (Tamura et al., 2007). The rRNA sequence was submitted to the database of GenBank with respective access number as described in Figure 1A.

*2.4. Collection of cell free supernatant*

Isolates of LAB were inoculated to 10mL of BHI broth and incubated at 37°C for 24h. Aliquots of the culture were transferred to 2mL polypropylene tubes, and centrifuged at 10,00 × *g* for 15min at 4°C. The resulting supernatant was neutralized (pH 7.0) with 1 mol $L^{-1}$ NaOH and heated at 95°C for 5min (Bromberg et al., 2006). This culture supernatant was used to evaluate the antimicrobial and antioxidant activity.

*2.5. Preparation of intracellular extract*

Isolates of LAB were inoculated in 10mL of BHI broth and incubated at 37°C for 24h. Aliquots of the culture were transferred to 2mL polypropylene tubes, and centrifuged at 10,000 × g for 15min at 4°C. The cell pellet was washed twice with Milli-Q water and resuspended in the same water followed by ultrasonic disruption. The sonication was performed in five intervals of 1 min in ice bath. Cellular debris was removed by centrifugation at 10,000 × *g* for 15min. The resulting supernatant was used as cell free extract to evaluate the antimicrobial and antioxidant activity.

*2.6. Antimicrobial activity*

The indicator microorganism, *L. monocytogenes* ATCC 7644, was suspended in 0.85% NaCl (w/v) standardized to optical density ($OD_{600}$) of 0.150 in spectrophotometer, which corresponded to a 0.5 McFarland turbidity standard solution. One aliquot of 20µL of culture supernatant was applied on cellulose discs (5 mm) onto BHI agar plates previously inoculated with a swab soaked in culture of the indicator bacterium. The plates were incubated at 37°C and inhibition zones were measured after 24h. The same procedure was performed to evaluate the antimicrobial activity of cell free extracts. The diameter of inhibition zones was measured using a caliper and halos ≥7mm were considered inhibitory (Bromberg et al., 2006). The experiment was performed in triplicate.

*2.7. Treatment of culture supernatant with proteases*

The sensibility of culture supernatant to protease was determined through sensibility of proteolytic enzyme. The proteases tested in this study were Proteinase K (Invitrogen) and Trypsin (Merck) dissolved in sterile phosphate buffer saline (PBS) at pH 7.4 in a concentration of 10mg $mL^{-1}$. Fifty microlitres of each culture supernatant was mixed with 50µL of both proteases (1:1) and incubated at 37°C for 1h. Enzyme inactivation was performed by means of heat treatment with boiling water for 10min, and after the samples were diluted with PBS buffer (1:1). Subsequently, aliquots of 20 µL were applied on cellulose discs (6 mm) onto BHI agar plates previously inoculated with a swab soaked in a culture of the indicator bacterium, *L. monocytogenes*. The plates were incubated at 37°C for 24h and then observed for the presence or absence of inhibition zones.

*2.8. Thiobarbituric acid reactive substances (TBARS)*

The reaction to thiobarbituric acid was performed according to the methodology of Ohkawa et al. (1979). Test tubes containing Milli-Q water, extra virgin olive oil were subjected to oxidation with 100µM ferrous sulfate and incubated in a water bath at 80°C, for 10min. Thereafter, to each tube was added the sample (culture supernatant and cell free extract of the bacteria), 81mg $mL^{-1}$ sodium lauryl sulfate (SDS), buffered acetic acid pH 3.44 and 6mg $mL^{-1}$ thiobarbituric acid (TBA). The reaction mixture was further incubated in a water bath at 100°C for 1h. For each sample tested had a blank to either the culture supernatant and cell free extract, and a standard control for all comparisons. The products of reaction were determined by measurement of absorbance at 532nm with a spectrophotometer. The concentration of TBARS was calculated using a standard curve developed with known concentrations of 1,1,3,3-tetramethoxypropane, and results were expressed as nmol of malonaldehyde (MDA)/mL of sample. The experiment was performed in triplicate.

*2.9. Antioxidant capacity using $ABTS^{\bullet+}$ method*

The antioxidant activity was determined using $ABTS^{\bullet+}$ (2,2 azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) radical cation (Re et al., 1999). $ABTS^{\bullet+}$ was dissolved in water (7m mol $L^{-1}$). ABTS radical cation ($ABTS^{\bullet+}$) was produced by reacting ABTS stock solution with 2.45m mol $L^{-1}$ potassium persulfate (final concentration) and allowing the mixture to stand in the dark at room temperature for 16 h before use. Stock solution was used for a maximum of 3 days. Before use, the $ABTS^{\bullet+}$ solution was diluted with ethanol, to an absorbance of 0.700±0.020 at 734nm.

Samples were diluted with ethanol to obtain between 20%-95% inhibition of the blank absorbance. Ascorbic acid was used as the standard in the range 0-9µg mL$^{-1}$. After addition of 10µL of sample (or standards) in 1.0 mL of ABTS$^{•+}$ solution, the absorbance was read at 30sec interval for 5min. Likewise, these same proportions (10µL of supernatant of culture medium or free extract and 1.0mL of ABTS$^{•+}$ solution) were used as a control.

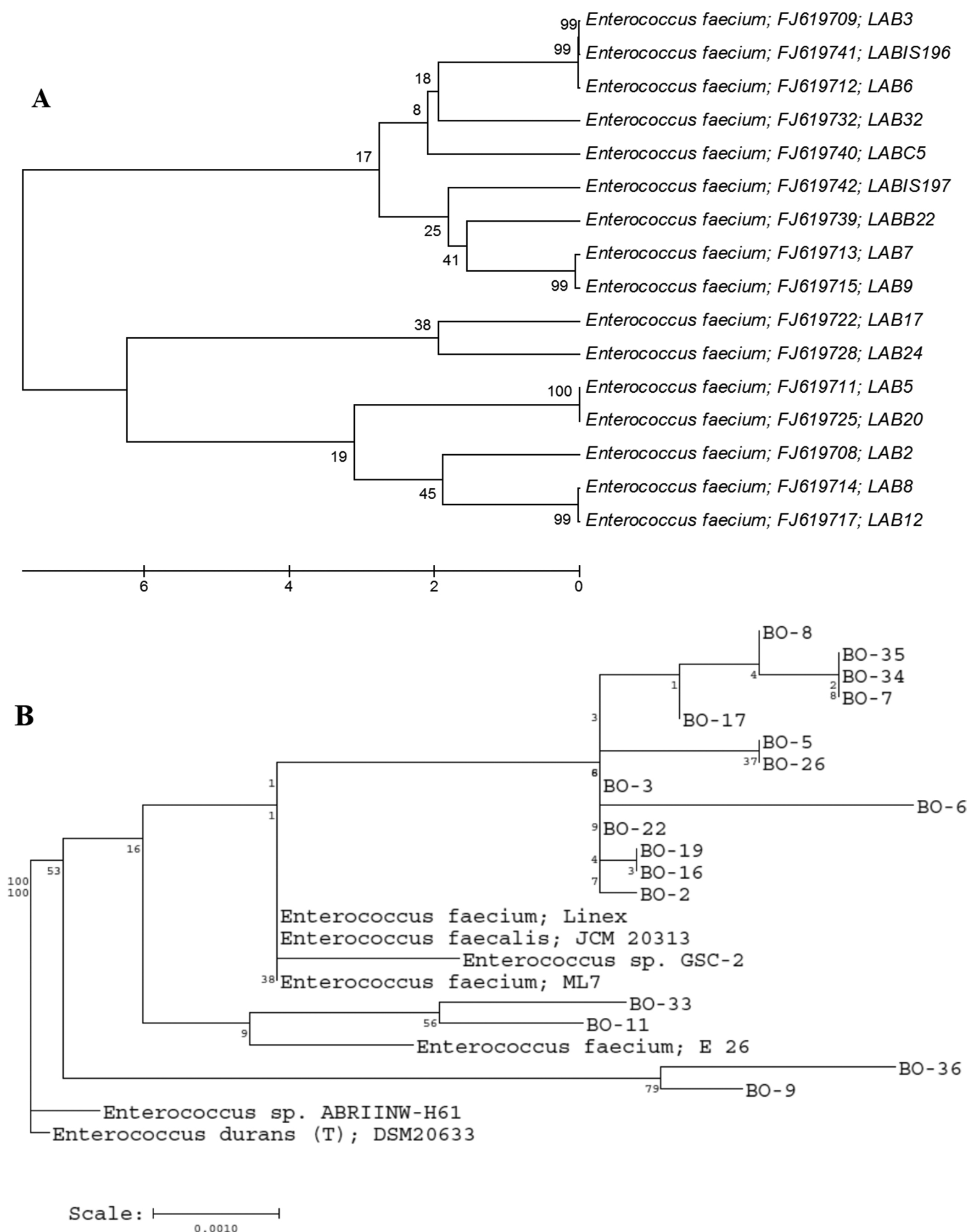


**Figure 1.** Phylogenetic tree (A) showing evolutionary distance between lactic acid bacterial isolates based on 16S rRNA gene sequence (500 bp). The scale represents the evolutionary distance value. The number at each node is the bootstrap from 100 replicates. (B) Phylogenetic tree showing evolutionary distance between lactic acid bacterial isolates from representative *Enterococcus* species using RDP release 10 software. LAB 2 (BO-2), LAB 3 (BO-3), LAB 5 (BO-5), LAB 6 (BO-6), LAB 7 (BO-7), LAB 8 (BO-8), LAB 9 (BO-9), LAB 12 (BO-11), LAB 17 (BO-16), LAB 20 (BO-19), LAB 24 (BO-22), LAB 32 (BO-26), LAB B22 (BO-33), LAB C5 (BO-34), LAB IS196 (BO-35), and LAB IS197 (BO-36). The scale represents the evolutionary distance value. The number at each node is the bootstrap from 100 replicates.

All determinations were carried out at least three times. The percentage inhibition of absorbance at 734nm was calculated using ascorbic acid standard curve.

*2.10. Scavenging ability on 2,2-diphenyl-1-picrylhydrazyl (DPPH) radicals*

The DPPH method used was as described by Brand-Williams et al. (1995) based on the capture of the DPPH (2,2-diphenyl-1-picrylhydrazyl) radical for antioxidants, producing a decrease in absorbance at 515nm. The DPPH was used at a concentration of 60μmol $L^{-1}$, dissolved in methyl alcohol. The solution was homogenized and transferred to a dark glass bottle. The prepared solution was used only in the day of analysis. In the dark, aliquots of 0.1mL of sample (culture supernatant or cell free extract) were transferred to test tubes with 3.9 mL of radical DPPH (60 μmol $L^{-1}$ DPPH solution) and homogenized by shaking. Likewise, these same proportions (0.1mL of culture medium or free extract and 3.9mL of radical DPPH) were used as a control. Methyl alcohol was used as a blank. The standard curve was DPPH in the range 0 to 60μmol $L^{-1}$. The results were expressed as $EC_{50}$ (μg $mL^{-1}$), which is the minimum antioxidant concentration required to reduce 50% of the initial DPPH reaction from the time the extract reached stability.

*2.11. Statistical analysis*

Means, standard deviation and standard error of replicates were performed by Excel program. Variance analysis were performed and significant results were compared by Tukey's test at $P<0.05$ with the Soc statistical Program (EMBRAPA, 1997) for TBARS and DPPH.

## 3. Results

*3.1. Bacterial identification*

All microorganisms were identified as Gram-positive and facultative anaerobic. The bacteria were identified by 16S rRNA sequence analysis as *Enterococcus faecium* (Figure 1). Blast analysis of 16S rRNA gene sequence revealed that isolates LAB 3, 6, 7, 9, 32, IS196 and IS197 presented 98% of similarity and the isolates LAB 2, 5, 8, 12, 17, 20, 24, and C5 had 99% of similarity (Figure 1A). In general, all isolates of LAB exhibited high similarity to *E. faecium* (Figure 1B). The isolates LAB 2, 3, 5, 6, 7, 8, 17, 24, 32, C5 and IS 196 (identified in the tree as BO, 2, 3, 5, 6, 7, 8, 16, 17, 19, 22, 26, 34 and 35, respectively) formed a group with high similarity to the *E. faecium* (Linex), and the bacteria *E. durans* (T) DSM20633 was the out group organism (Figure 1B).

*3.2. Antimicrobial activity*

Antimicrobial activity of culture supernatants and cell free extracts of the isolates are summarized in Table 1. Of the 16 isolates analyzed, 11 isolates displayed inhibitory activity against the indicator organism *L. monocytogenes* when the culture supernatant was used, and 4 isolates showed inhibitory activity with the cell free extract. Inhibitory capacity of the culture supernatants of LAB isolates against *L. monocytogenes* (Table 1) ranged from 7.0 to 11.0mm. The highest inhibition zones, 10.7mm and 11.0mm were respectively observed with culture supernatants of isolates IS196 and IS197. The lowest zones of inhibition were recorded with culture supernatants of isolate LAB 24 (7.0mm) and cell free extracts of isolate LAB 32. Antimicrobial activity against *L. monocytogenes* was observed in both culture supernatants and cell free extracts of LAB isolates 17, 32, IS196 and IS197. Other isolates displayed only extracellular antimicrobial activity.

**Table 1.** Antimicrobial activity of culture supernatants and cell free extracts of LAB against *Listeria monocytogenes* ATCC 7644.

| Strain | Inhibition zone (mm) | |
|---|---|---|
| | Culture Supernatant | Cell Extracts |
| LAB 2 | 9.0±0.32* | 0.0±0.00 |
| LAB 3 | 7.7±0.15 | 0.0±0.00 |
| LAB 5 | 8.0±0.12 | 0.0±0.00 |
| LAB 6 | 0.0±0.00 | 0.0±0.00 |
| LAB 7 | 7.3±0.06 | 0.0±0.00 |
| LAB 8 | 0.0±0.00 | 0.0±0.00 |
| LAB 9 | 0.0±0.00 | 0.0±0.00 |
| LAB 12 | 0.0±0.00 | 0.0±0.00 |
| LAB 17 | 8.3±0.09 | 8.3±0.17 |
| LAB 20 | 0.0±0.02 | 0.0±0.00 |
| LAB 24 | 7.0±0.02 | 0.0±0.00 |
| LAB 32 | 8.0±0.18 | 7.0±0.02 |
| LAB B22 | 8.0±0.08 | 0.0±0.00 |
| LAB C5 | 7.5±0.16 | 0.0±0.00 |
| LAB IS196 | 10.7±0.13 | 9.7±0.23 |
| LAB IS197 | 11.0±0.15 | 9.3±0.08 |

*Results are means ± standard error of three independent experiments.

The sensibility of antimicrobial activity to proteinase K and trypsin was evaluated. The inhibitory substance(s) produced by *E. faecium* isolates were inactivated by both proteolytic enzymes tested. After treatment with proteinase K and trypsin, the antimicrobial activity was completely lost. The control supernatants submitted only to heat treatment maintained 100% their initial activities.

*3.3. Antioxidant activity*

The antioxidant activity of both culture supernatants and cell free extracts were evaluated by different methods: TBARS, $ABTS^{\bullet+}$ radical cation, and DPPH methods.

3.3.1. Thiobarbituric acid reactive substances (TBARS)

Both culture supernatants and cell free extracts of all LAB isolates used in this study showed antioxidant activity by the TBARS method. However, some preparations showed higher activity compared to the control, particularly culture supernatants of the *E. faecium* isolates 6, 7, 9, IS196 and IS197 (Figure 2A). The cell free extracts of the isolates LAB 3, 6, 8, B22, C5, IS196 and IS197 showed higher antioxidant activity (Figure 2B).

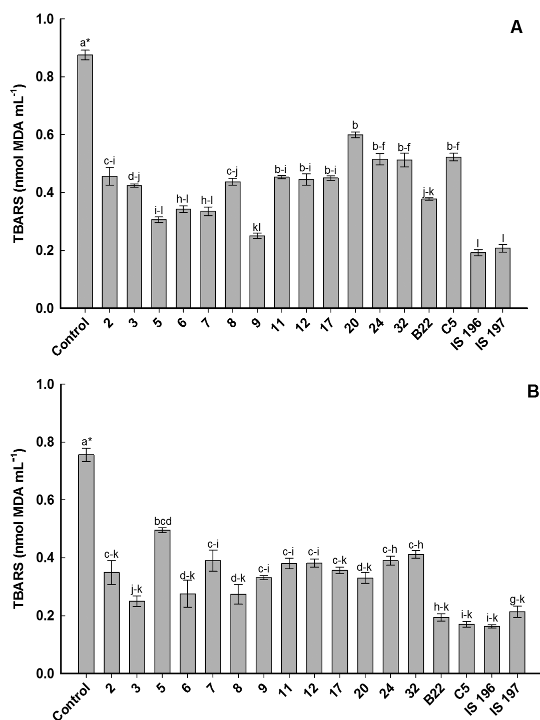


**Figure 2.** Evaluation of antioxidant activity of culture supernatants (A) and cell extracts (B) of isolates using thiobarbituric reactive substances (TBARS) method. Results are means ± standard error of three independent experiments. *Different letters are significant different with 0.05 P by Tukey test. Letters separated from "-" means that are continuous, i.e. "d-k" read "defghijk".

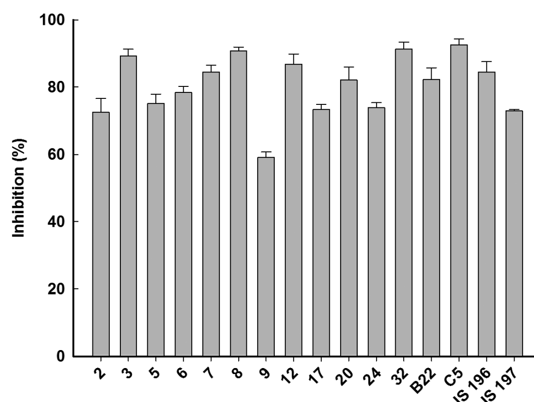


**Figure 3.** Determination of antioxidant capacity of culture supernatants using the $ABTS^{•+}$ method. Results are means ± standard error of three independent experiments.

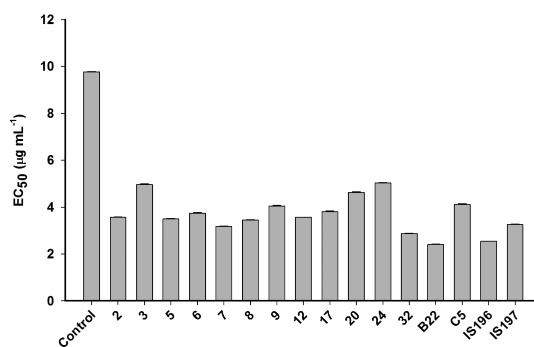


**Figure 4.** Determination of antioxidant capacity of culture supernatants using the DPPH method. Results are means ± standard error of three independent experiments.

3.3.2. $ABTS^{•+}$ free radical scavenging assay

All samples of the culture supernatants showed ability to sequester free radicals by the $ABTS^{•+}$ method (Figure 3). Isolates LAB 3, 8, 12, 32, and C5 showed higher percentage of inhibition and consequently, higher antioxidant activity when compared to the other *E. faecium* isolates, which showed inhibitory activity between 59 and 92.5%. Contrarily, samples of cell free extracts showed weak antioxidant activity with percent inhibition between 0.6 and 6% for *E. faecium* isolates (data not shown).

3.3.3. Assay for DPPH radical-scavenging activity

The results obtained with the culture supernatants are shown in Figure 4. All isolates showed antioxidant activity compared with the control ($EC_{50}$=9.77µg $mL^{-1}$). $EC_{50}$ values ranging from 2.41-5.02µg $mL^{-1}$ were observed with the *E. faecium* culture supernatants. Strain B22 displayed the lowest $EC_{50}$ value (2.41µg $mL^{-1}$) and, consequently, higher antioxidant activities in terms of DPPH free radical scavenging. The results of cell extracts of enterococci showed no antioxidant activity measured by the DPPH free radical scavenging activity method. However, the cell extract of one strain, *E. faecium* strain 3, displayed significant antioxidant activity with an $EC_{50}$ of 7.9µg $mL^{-1}$ (data not shown).

## 4. Discussion

Antimicrobial and antioxidant activities of LAB are of extreme importance in fermented foods. In this study, both antioxidant and antimicrobial activities were investigated in culture supernatants and cell free extracts of lactic acid bacteria isolated from regional dairy and meat products. The selected isolates were identified as *Enterococcus faecium*, which is a Gram-positive, catalase-negative cocci that share many characteristics with the genera *Lactococcus* and *Streptococcus* (Valenzuela et al., 2009). The enterococci are a complex and important group of bacteria that can be found in a variety of food products, such as milk and cheese, meat and vegetables (De Vuyst et al., 2003; Gomes et al., 2008).

The small fragment (500 bp) of the 16S rRNA gene was used to characterize the microbial community in this study. This region is called V3 region. The V3 region of 16S rRNA gene is a commonly used region for bacterial phylogenetic analysis. Despite of the short nucleic acid sequences, hypervariable V3 region can provide information enough to describe microbial community (Liu et al., 2007). The isolates were clustered within *E. faecium* species, and the results obtained by 16S rRNA sequencing indicated a

high degree of sequence similarity among them and from a phylogenetically coherent group of lactic acid bacteria. Also, LAB isolates were designated to the correct species with close homology. In fact, BLAST search analyses using 16S rDNA sequence resulted in homologies between 96 and 99%. Interestingly, *Enterococcus* species are frequently found in traditional fermented foods and may be included as a component of some mixed *starters* (Vasiljevic and Shah, 2008).

Antimicrobial activity against *L. monocytogenes* was observed in 70% of the culture supernatants of the tested isolates, indicating that antilisterial substances are secreted by these bacteria. *L. monocytogenes* can tolerate a wide range of pH, temperatures, salt concentration and water activity that can be undesirable to many other bacteria. In ready-to-eat products, refrigeration is the principal method to control undesirable microorganisms in many cases and sometimes the only method of preservation. However, some psychrotrophic pathogenic microorganisms, like *L. monocytogenes*, can multiply with little or no change in sensory characteristics of products. Therefore, the inhibition of *Listeria* is very relevant to food safety since this pathogen has been associated with several disease outbreaks (Gandhi and Chikindas, 2007; Shen et al., 2013).

Antilisterial activity has been described for *Enterococcus* spp., which may synthesize several antimicrobial substances, such as organic acids, hydrogen peroxide, carbon dioxide, diacetyl, acetaldehyde and bacteriocins, called enterocins (Naidu et al., 1999). Although the nature of the antimicrobial substances was not determined in this study, treatment with the enzymes proteinase K and trypsin resulted in the inactivation of the antimicrobial activity. Thus, it seems that the antimicrobial compounds produced by *E. faecium* isolates are a thermostable protein or peptide showing antilisterial activity, resembling enterocins. Production of enterocins is widespread among *Enterococcus* spp. (Maqueda et al., 2008). Although enterocins are most frequently produced by *E. faecium* isolates, many other species of *Enterococcus* have also been found to produce bacteriocins, including *E. faecalis*, *E. hirae, E. mundtii, E. durans, E. avium, E. gallinarum, E. casseliflavus* and *E. columbae* (Sabia et al., 2004; Sánchez et al., 2007).

Several methods have been developed to evaluate antioxidant activity, including quantification of products formed during lipid peroxidation (TBARS), free radicals ($ABTS^{\bullet+}$ and DPPH) scavenging assays and superoxide dismutase activity (Sanchez-Moreno et al., 1998; Frankel and Meyer, 2000; Aruoma, 2003). In this work, the *in vitro* antioxidant activity of the food isolated enterococci was observed by different methods, although the radical scavenging effect was only observed for culture supernatants. On contrast, free radical scavenging activity was described to intracellular extracts of some LAB, such as *Lactobacillus delbrueckii* (Lin and Yen, 1999), *Lactobacillus acidophilus* and *Bifidobacterium longum* (Lin and Chang, 2000).

Olive oil (monounsaturated fatty acid) was used in TBARS method as substrate and ferrous sulfate as a pro-oxidant since it can split lipid hydroperoxides. The oxidation of olive oil was inhibited by adding the samples of culture supernatants and cell free extracts, as a clear decrease in absorbance was observed resulting from the inhibition of lipid peroxidation (antioxidant activity). These results suggest that the isolates have antioxidant properties.

Although TBARS method is widely accepted, other methods for evaluation of antioxidant activity, like DPPH radical method and $ABTS^{\bullet+}$ radical method, can be useful. These tests are different in relation to reaction mechanism to target radical species, reaction conditions and expression of the results. There is no universal method to evaluate antioxidant activity, thus it is necessary to use different methods to properly evaluate the antioxidant capacity (Huang et al., 2005). The antioxidant activity of culture supernatants and cell extracts were measured as capability of sequestration of free radicals, according to the $ABTS^{\bullet+}$ method. This method measures the ability of the sample in sequester the radical $ABTS^{\bullet+}$, compared with a standard amount of Trolox (Trolox Equivalent Antioxidant Capacity), and it is an excellent tool to determine the antioxidant activity of antioxidants and hydrogen donors terminators of antioxidants chains (Re et al., 1999).

The high antioxidant activity found in culture supernatants by $ABTS^{\bullet+}$ method was confirmed by the results obtained with the DPPH radical method. The DPPH free radical is stable but efficient antioxidant substances transfer electrons or hydrogen atoms to it neutralizing its radical character (Naik et al., 2003). Microbial antioxidants are involved in termination of free radical reactions and reducing power (Yang et al., 2000). The DPPH test provides information about the reactivity of an antioxidant with a stable free radical (Banerjee et al., 2005).

Absence of antioxidant activity in cell extracts of some isolates suggests that this property may be extracellular. It may also be due to loss of activity from the method of extraction. The effectiveness of ultrasonic disruption of microbial cells varies between organisms, and the influence of ultrasonic waves in the activity and stability of enzymes has been shown to be specific for each enzyme and dependent on parameters of sonication (Özbek and Ülgen, 2000). Detection of the antioxidant activity of these microorganisms in the culture supernatant offers a practical advantage in that it eliminates the need for free radical transport to cellular sites with antioxidant activity.

In summary, the antimicrobial and antioxidant capacities of these *E. faecium* isolates indicate they could be very useful in food fermentation and feed composition. They could particularly help to inhibit pathogenic microorganisms as well as oxidative spoilage in foods and feeds.

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