Assessment of the bioprotective potential of lactic acid bacteria against *Listeria monocytogenes* in ground beef

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Abstract

Lactic acid bacteria can be considered as natural biopreservative and good biotechnological alternative to food safety. In this study, the antilisterial compounds produced by *Enterococcus* isolates from the Patagonian environment and their effectiveness for the control of *Listeria monocytogenes* in a food model were studied. *Enterococcus* isolates whose cell-free supernatant presented activity against *Listeria monocytogenes* were identified and evaluated for their virulence factors. The activity of the antimicrobial compounds produced by *Enterococcus* sp. against *Listeria monocytogenes* Scott A in meat gravy and ground beef during refrigerated storage was tested. The results indicated that ten *Enterococcus* isolates presented activity against *Listeria monocytogenes* and none of the selected strains presented virulence factors. *L. monocytogenes* in the food models containing the antilisterial compounds produced by *Enterococcus* sp. has decreased over the days, indicating that these compounds and cultures are an alternative to control the growth of *L. monocytogenes* in foods.

Keywords  *Enterococcus mundtii* · *Listeria monocytogenes* · Antimicrobial activity · Meat model · Food safety

Introduction

Foods can be contaminated by microorganisms such as *Listeria monocytogenes*, *Pseudomonas* spp., among others and/or bacterial microbiota and the main problem in the food industry is how to prevent and eliminate the presence of pathogenic bacteria through cleaning and disinfection mechanisms, that are sometimes inefficient. Furthermore, the use of chemical agents for food preservation is not compatible with the image of minimally processed fresh products. Hence, the presence of lactic acid bacteria (LAB) is of great interest since they are Generally Recognized As Safe (GRAS) (Dal Bello et al. 2012) and they can be considered as biopreservative cultures (Stiles 1996). LAB may be used in food preservation because they compete with other microorganisms and produce compounds with antimicrobial activity such as lactic acid, acetic acid, hydrogen peroxide, diacetyl and ethanol (Leroy and De Vuyst 2004; Tomé et al. 2006). Moreover, many LAB strains are capable of producing bacteriocins in foods (Cotter et al. 2005; Panesar et al. 2007) that may inhibit the growth of pathogenic Gram-positive bacteria, as well as some yeasts and Gram-negative species (Kailasapathy 2006).

Among the LAB, *Enterococcus* is one of the genera and some strains of *Enterococcus* have health-promoting properties and a potential technological advantage in the food industry. They present interesting enzymatic activities and acidifying, reducing and proteolytic properties that improve their sensory quality, contribute to the texture of foods, aroma and flavor (Giraffa 2003; Ogier and Serro 2008). In addition, many *Enterococcus* strains produce bacteriocins that give them an advantage over pathogenic...
bacteria or the microbial flora of food. Therefore, *Enterococcus* could be used as natural biopreservative cultures to improve food safety.

The Patagonian marine environment has its own physical and chemical characteristics that allows the isolation of *Enterococcus* with particular physiological properties (Marguet et al. 2011). High salinity and low temperatures exert a selective pressure that results in a microbiota that has been little studied, but nevertheless presents a biotechnological potential of interest, both for the study of the production of metabolites and for the use of *Enterococcus* strains.

The presence of safety factors (hemolytic and gelatinase activities, virulence factors and antibiotic resistance) must be investigated because the safety evaluation of enterococcal strains and the use as biopreservative cultures is very important (Ben Braïek et al. 2018). The bacteriocinogenic strain should be able to grow and produce bacteriocins in situ or ex situ, to present bacteriocin diffusion through food and the parameters that influence metabolic production, and the use of LAB as bioprotective cultures should not change the sensory characteristics in foods (Dicks et al. 2004).

The objective of this work was to report the partial characterization of bacteriocins produced by Patagonian *Enterococcus* strains, to evaluate the virulence factors of the selected strains, and their potential use as biopreservative cultures/antilisterial extract) against *Listeria monocytogenes* in a food model during refrigerated storage (4 °C) for 15 days.

### Material and methods

#### Indicator strains and culture conditions

In Table 1, the target strains used in the study are listed. LAB strain isolated from the Patagonian area was inoculated in MRS broth (Biokar Diagnostics, Beauvais, France) and

| Indicator strains | Growth media | Antimicrobial activity |
|-------------------|--------------|-----------------------|
|                   | Temperature (°C) | *E. mundtii Tw56* | *E. mundtii Tw802* | *E. mundtii Tw807* |
| *Enterococcus faecalis* ATCC 29,212 | MRS, 37 °C | + + | + + | + + |
| *Enterococcus Van A* | MRS, 37 °C | + + | + + | + + |
| *Enterococcus Van B* | MRS, 37 °C | + + | + + | + + |
| *Enterococcus Van C* | MRS, 37 °C | + + | + + | + + |
| *Enterococcus casseliflavus* | MRS, 37 °C | + + | + + | + + |
| *Enterococcus faecium* | MRS, 37 °C | + + | + + | + + |
| *Escherichia coli ATCC 25,922* | TS, 35 °C | – | – | – |
| *Escherichia coli ATCC 35,218* | TS, 35 °C | – | – | – |
| *Lactococcus lactis* ATCC 11,454 | MRS, 30 °C | – | – | – |
| *Lactococcus garvieae* 03/8460 | MRS, 30 °C | – | – | – |
| *Lactococcus garvieae* 03/8702 | MRS, 30 °C | – | – | – |
| *Lactococcus piscium* 23.3.92 | MRS, 30 °C | – | – | – |
| *Lactobacillus plantarum* TwLb 5 | MRS, 30 °C | + | + | + |
| *Listeria innocua* Tw 67 | TS, 30 °C | + + + | + + + | + + + |
| *Listeria innocua* 6a | TS, 30 °C | + + + | + + + | + + + |
| *Listeria innocua* ATCC 33,090 | TS, 30 °C | + + | + + | + + |
| *Listeria monocytogenes* Scott A | TS, 30 °C | + + + | + + + | + + + |
| *Listeria monocytogenes* ATCC 7644 | TS, 35 °C | + + + | + + + | + + + |
| *Listeria monocytogenes* 1908a | TS, 35 °C | + + + | + + + | + + + |
| *Listeria monocytogenes* 1915a | TS, 35 °C | + + + | + + + | + + + |
| *Listeria monocytogenes* 1599a | TS, 35 °C | + + + | + + + | + + + |
| *Micrococcus luteus* ATCC 15,307 | TS, 30 °C | – | – | – |
| *Streptococcus iniae* MT 2376 | MRS, 37 °C | – | – | – |
| *Pseudomonas aeruginosa* ATCC 27,853 | TS, 30 °C | – | – | – |
| *Yersinia ruckeri* 02/1607/C | TS, 25 °C | – | – | – |

Diameter of clear zone; + , ≥ 10 mm; + + , ≥ 15 mm; + + + , ≥ 20 mm; – , no inhibition

*Strains provided by Lic. Ledesma (Fac. de Cs. Naturales y Cs. de la Salud, Universidad Nacional de la Patagonia San Juan Bosco)

*Strains provided by Dr. Fernandez-Garayzabal (Escuela de Medicina Veterinaria, Universidad Complutense de Madrid)
was grown at 25 °C for 18 h, and the other bacteria were inoculated in BHI broth (Difco, Le Pont de Claix, France) and were grown at 37 °C for 24 h. Cell-free supernatants (CFS) were separated to evaluate the antimicrobial activity. LAB strain was grown in MRS broth at 30 °C for 18 h and was centrifuged at 8000 rpm (for 15 min at 4 °C) in a high-speed centrifuge (Hettich Zentrifugen, model Mikro 22R, Germany). The CFS obtained was treated at 100 °C for 5 min, was filtered through a 0.20 μm pore sterilizing syringe filter (Sartorius, Stedim Biotech, Germany) and was stored at −20 °C. Antimicrobial activities of the LAB strains isolated were evaluated by the double-layer diffusion test, according to Farías et al. (1994), using *Listeria innocua* ATCC 33,090 and *Listeria monocytogenes* Scott A as indicators, and the results were expressed as arbitrary units per milliliter (AU mL−1). Nisin (1000 UI mL−1, Nisaplin, Danisco, Denmark) and distilled water were used as positive and negative controls, respectively.

**Isolation, phenotypic and genetic identification of LAB strains**

The LAB strains were isolated from the provinces of Chubut and Santa Cruz (between −42.00 latitude and −52.00 latitude) of the VIRCH-Valdes and Río Senguer-Golfo San Jorge regions (Patagonia, Argentina). The isolates, as well as their phenotypic identification, were made in MRS agar medium (Biokar) as described by Vallejo et al. (2009). Three isolates were selected to be identified by 16S rDNA amplification and sequencing as described by Delcarlo et al. (2019).

**Determination of virulence factors**

**Evaluation of strain safety and PCR for the detection of virulence factors**

The production of gelatinase, hemolysin and exopolysaccharides was carried out as described by Delcarlo et al. (2019). The susceptibility of *Enterococcus* strains to penicillin, ampicillin, amoxicillin/clavulanic, erythromycin, rifampin, vancomycin trimethoprim/sulfamethoxazole, and teicoplanin was evaluated according to the National Committee for Clinical Laboratory Standards (NCCLS 2015) guidelines. Extracted DNA was used as a template for the amplification of virulence genes and the general PCR conditions were those described by the authors in Table 2.

**Characterization of antimicrobial activity**

**Determination of the active principle causing the inhibitory activity**

The CFSs were fractionated into three aliquots. The first CFS was called “crude”. The second CFS was neutralized to pH 6.5 with 0.5 M NaOH (called “neutralized CFS”) and the third CFS “heated” was neutralized and heated at 100 °C for 5 min. Then, the above preparations were evaluated as Vallejo et al. (2009).

The CFSs were incubated at 37 °C for 1 h with catalase (2 mg mL−1), trypsin, proteinase K, lipase or lysozyme (Sigma, USA) in a final concentration of 1.0 mg mL−1 (Vallejo et al. 2009). Enzyme solutions were diluted with sterile water and used as negative and positive controls, respectively. The residual antilisterial activity was determined according to Farías et al. (1994).

| Table 2 PCR amplification of potential enterococcal virulence factors |
|---------------------------------------------------------------|
| Virulence Factor | Sequence (5′–3′)                                      | Size (pb) | Reference             |
|------------------|------------------------------------------------------|-----------|-----------------------|
| Agg              | f: AAGAAAAAGTAGAGCAAC                              | 1553      | Eaton and Gasson (2001) |
|                  | r: AAACCGCAAGCAAGTAAAAT                              |           |                       |
| gelE             | f: ACCCGTATTTTGTTT                                  | 419       | Eaton and Gasson (2001) |
|                  | r: ACCGATTGCTTTTCCATC                                |           |                       |
| Esp              | f: TTGCTAATGGCTAAGCAGACC                            | 933       | Eaton and Gasson (2001) |
|                  | r: GCCTGACCACCTTGCTAGGCAAGA                         |           |                       |
| hylEfms          | f: GAGTAGAGGAATATCTTAGC                              | 661       | Rice et al.(2003)     |
|                  | r: AGGTCCATTTCTGT                                   |           |                       |
| IS16             | f: CATGTTCCACGAGACAG                                | 547       | Werner et al. (2011)  |
|                  | r: TCAAAAAAGTGGCCTTGCG                              |           |                       |
| ClyL4            | f: GATGGAGGTAAAGATTAGG                               | 253       | Semedo et al. (2003)  |
|                  | r: GCTTACCTCAATAAGTTTTAG                             |           |                       |
| ClyLs            | f: GAAGCCACAGTGCTAAATA AGG                          | 240       | Semedo et al. (2003)  |
|                  | r: GTATAAGGGCTATTTTCA                               |           |                       |
Stability test for antimicrobial compounds produced by Enterococcus sp.

The influence of temperature on CFSs activity was evaluated by keeping the CFSs at 40–100 °C for 30 min and then the activity against L. monocytogenes Scott A was observed. Different pH treatments ranging from 2 to 10 were used to evaluate the residual antilisterial activity. The mixture was incubated for 2 h at 25 °C and residual antilisterial activity was evaluated.

PCR screening for enterocin structural genes

The presence of structural genes of bacteriocins present in the genome of the bacteria studied was carried out by PCR and the primers used for the amplification of enterocin A, B, P, L50A, L50B, mundticin KS, bacteriocina 96, bact 31, 1071 A/B, enterocin Q and HirJM 79 and corresponding references are listed in Table 3. General PCR conditions were described by Schelegueda et al. (2015).

Bacteriocin production from LAB in different culture media and incubation conditions

The culture media employed for bacteriocin production were: MRSs broth (Biokar), MRSc (Biokar), LAPTgs (Raibaud et al. 1963) and LAPTgc. Subscripts s and c denote without or with cysteine (Sigma, USA) (0.5 mg mL⁻¹), respectively. Incubation temperatures were 25, 30 and 37 °C, without or with agitation (100 rpm). The CFSs were neutralized (pH 6.5) and the antilisterial activity quantification was performed by the critical dilution technique (Rosa et al. 2002).

Determination of antimicrobial spectrum and quantification of antimicrobial activity

Since E. mundtii Tw56, Tw802 and Tw807 had the highest antimicrobial activity against L. monocytogenes, they were selected to determine their spectrum of activity against other strains that cause food spoilage (Table 1). The antimicrobial activity of neutralized CFSs against the indicator microorganism was performed according to Faria et al. (1994). The formation of inhibition halos in the plates was evaluated after 18 h at 30 °C.

Evaluation of bacteriocinogenic strains against Listeria monocytogenes Scott A in food models

The protective effect of E. mundtii Tw56, Tw802 and Tw807 against L. monocytogenes Scott A was evaluated either by

Table 3 Primer sequences for PCR amplification of enterocin genes in Enterococcus mundtii Tw56, Tw802 and Tw807

| Enterocin | Sequence (5´–3´) | Size (pb) | Reference |
|-----------|------------------|-----------|-----------|
| Ent A     | f: GGTACCACTCATAGTGGAAA r: CCCTGGAATTGCTCCACCTAA | 138 | De Vuyst et al. (2003) |
| Ent B     | f: CAAAATGTAAAGAATTAAGTACG r: AGAGTATACATTTGCTAACC | 201 | De Vuyst et al. (2003) |
| Ent P     | f: GCTACCGGTTCATATGTGAAT r: TCCTGCAAAATATCCTTACC | 87 | De Vuyst et al. (2003) |
| Ent A/B50A| f: ATGGGAGCAGATCGAAATAA r: TTTGTAAAATGCCCCATCCTTC | 274 | De Vuyst et al. (2003) |
| Ent A/B50B| f: ATGGGAGCAATCGAATAATA r: TAGCCATTTTCTAATTTGATC | 274 | De Vuyst et al. (2003) |
| Bact 96   | f: GTGGAGAGGACGAAAGGAGA r: TTGGATTACGGAGACGCGATTAA | 291 | Henning et al. (2015) |
| Bact 31   | f: CCTACGTATACGGCAATTGTT r: GCCATTTGTCACCAACATT | 130 | Özdemir et al. (2011) |
| 1071 A/B  | f: GGGGAGATCGGTGTGTTT TAG r: ATCATATGCAGGTTGACCC | 273 | Martín et al. (2006) |
| EntQ      | f: ATGAAATTTTTCTCTTCCTAAAATGTATCGCA r: TAAACAGAAATTTTTCTCCATGGCACA | 105 | Belgacem et al. (2010) |
| mun KS    | f: TGAGAGAGGTGTTAAGTGGTAAGA r: TCCACCTGAAATCCGGATGA | 379 | Zendo et al. (2005) |
| HirJM 79  | f: ATGAAAAAGGAAATTTAATACATTGTGTTATCTACG r: ATAAAGTAAAGCCTGTACCTACCTTACCTAGGTGCCCATGGACC | 408 | Almeida et al. (2011) |
direct application or incorporation of the neutralized antilisterial extract (Yang et al. 1992) in meat gravy and ground beef models. The food models were divided into 14 fractions. Each one (A–N), received the following treatment: A) control, L. monocytogenes Scott A (10^4 CFU mL⁻¹ or g⁻¹); B, C and D) control, E. mundtii Tw56, Tw802 or Tw807 (10^7 CFU mL⁻¹ or g⁻¹), respectively; F, G and H) control, antilisterial extract (10^4 AU mL⁻¹ or g⁻¹) produced by E. mundtii Tw56, Tw802 or Tw807, respectively; I, J and K) L. monocytogenes Scott A (10^4 CFU mL⁻¹ or g⁻¹) and E. mundtii Tw56, Tw802 or Tw807 (10^7 CFU mL⁻¹ or g⁻¹), respectively and L, M and N) L. monocytogenes Scott A (10^4 AU mL⁻¹ or g⁻¹) and antilisterial extract (10^4 AU mL⁻¹ or g⁻¹) produced by E. mundtii Tw56, Tw802 or Tw807, respectively.

Meat gravy model

A simulated meat gravy model was prepared mixing proteose peptone (1.8% w v⁻¹), meat extract (1.2% w v⁻¹), yeast extract (0.6% w v⁻¹) and corn starch (2.0% w v⁻¹) and the experiments were performed as described by Carvalho et al. (2018). The inoculated gravy portions were refrigerated at 4 °C for 15 days. For counting, the gravy portions were submitted to serial decimal dilutions in 0.85% NaCl (w v⁻¹) solution and 100 μL of each dilution were spread plated onto two plates of Oxford agar (Biokar) for the enumeration of L. monocytogenes Scott A and two plates of MRS agar (Biokar) for enumeration of Enterococcus strains. Plates were incubated at 37 °C for 48 h, the average number of colonies was calculated, and the results were expressed as log CFU mL⁻¹ or g⁻¹. For counting, the gravy portions were submitted to serial decimal dilutions in 0.85% NaCl (w v⁻¹) and corn starch (2.0% w v⁻¹) and the experiments were performed as described by Carvalho et al. (2018). Inoculated gravy portions were refrigerated at 4 °C for 15 days. For counting, the gravy portions were submitted to serial decimal dilutions in 0.85% NaCl (w v⁻¹) solution and 100 μL of each dilution were spread plated onto two plates of Oxford agar (Biokar) at 30 °C for 48 h, the average number of colonies was calculated, and the results were expressed as log CFU mL⁻¹ or g⁻¹.

Ground beef model

Ground beef burgers from the muscles of the quadriceps femoris obtained from a local butcher’s shop were prepared as described by Acuña et al. (2015). They were refrigerated at 4 °C in sterile plates for 15 days. Samples of each condition were evaluated on days 0, 1, 3, 5, 7, 10 and 15.

Food model samples were diluted using sterile saline (0.85%) as diluent and 100 μL of each dilution were surface-plated onto Oxford agar (Biokar) to count L. monocytogenes Scott A and onto MRS agar (pH 5.5) (Biokar) to enumerate Enterococcus strains. All plates were incubated at 37 °C for 48 h, the average number of colonies was calculated, and the results were expressed as log CFU mL⁻¹ or g⁻¹. In addition, a total mesophilic control was performed in the ground beef, using Agar Plate Count (Biokar) at 30 °C for 48 h. All experiments were done three times. In addition, the production of bacteriocins was evaluated by the methodology before mentioned (Farías et al. 1994).

Data analysis

Variance analysis (ANOVA) was applied to the results of microbial enumeration in simulated food models (Carvalho et al. 2018).

Results and discussion

Strain selection and safety evaluation

The present study evaluated 9 LAB strains (E. mundtii Tw56, E. mundtii Tw222, E. mundtii Tw278, E. faecium Tw452, E. faecium Tw465, E. faecalis Tw471, E. mundtii Tw492, E. mundtii Tw802 and E. mundtii Tw807), isolated from marine invertebrates (sea cucumber, mussel, marine snail) and E. faecium Tw6, isolated from sheep’s milk. Their CFSs presented a potent inhibitory activity on L. innocua ATCC 33,090, L. monocytogenes Scott A and other indicator strains (Table 1). Three strains produced inhibition halos larger than 20 mm in diameter, identified as E. mundtii Tw56, E. mundtii Tw802 and E. mundtii Tw807, were selected for use as a biopreservative potential in food model. The results obtained in this study indicated that the selected Enterococcus strains do not present potential virulence factors, gelatinase activity (no clear zones) in agar plates and resistance to antibiotic.

The demand for minimally processed and safe food products means highlighting the search for new preservation methods. In this study, characterized LAB strains and their CFSs presented a potent inhibitory activity on L. innocua ATCC 33,090, L. monocytogenes Scott A and other indicator strains. The bacteriocins produced by Enterococcus sp. were evaluated because of their antagonistic activity against pathogenic microorganisms (De Kwaadsteniet et al. 2005). These microorganisms have been removed from FAO’s GRAS list because they are associated with foodborne diseases (Khan et al. 2010), but Enterococcus strains do not generally have virulence factors and may be a suitable LAB to be applied in food preservation (Franz et al. 2003, Giraffa 2003, Schelegueda et al. 2016). Gelatinase is an important virulence factor present in isolates from food, environmental and clinical specimens (Eaton and Gasson 2001). Another enterococcus virulence factor is the presence of hemolysin, because it is the cause of enterococcal disease in humans and animals (Semedo et al. 2003). Hemolytic activity in human blood agar and exopolysaccharide production were not observed in all Enterococcus strains studied in this work. There were no halos of complete hemolysis around the colonies and no black colonies of the selected strains and the absence of virulence genes was observed by
PCR techniques. Since the genus *Enterococcus* has caused great concern due to its increasing resistance to antimicrobial agents, that of the different strains to vancomycin and other antibiotics was evaluated. None of the strains showed resistance to penicillin, ampicillin, amoxicillin/clavulanic, erythromycin, trimethoprim/sulfamethoxazole, teicoplanin or vancomycin and only the strain *E. mundtii* Tw807 exhibited resistance to rifampicin. Before using enterococci in foods their safety, as well as their functional and beneficial traits, should be carefully evaluated. The strains should be phenotypically and genotypically characterized and ought to be free from any pathogenicity and virulence factors (Braïek and Smaoui 2019).

**Characterization of antimicrobial activity**

The characterization of antilisterial compounds secreted by *Enterococcus* strains confirmed they were sensitive to proteolytic enzymes, evidencing the proteinaceous nature. The CFSs were thermostable after heating at 100 °C for 30 min and active in a broad pH range (between 2 and 10). Antimicrobial activity was not changed by the treatment with lipase or catalase, demonstrating that neither lipids nor hydrogen peroxide were involved in the inhibitory activity. Bacteriocin sensitivity to different enzymes is used for the identification of potential cultures in the control of foodborne pathogens, as observed in Barbosa et al. (2014), Carvalho et al. (2018) and Decarlo et al. (2019). The selected strains of *Enterococcus* sp in this work were described as bacteriocin or bacteriocin-like substance producers and their possible applicability as biopreservative in the food industry. The antagonistic activity was not lost after treatment with trypsin, showing a protein composition of the antimicrobial substances and neither after treatment with lysozyme that acts on glycosidic bonds (Decarlo et al. 2019).

PCR amplifications were performed on the genomic DNA of *E. mundtii* Tw56, *E. mundtii* Tw802 and *E. mundtii* Tw807 to find out the presence of structural bacteriocin genes in the genome of the selected strains. The structural gene of enterocin KS was amplified in the genome of the three strains. The structural gene of enterocin B was present in the strains *E. mundtii* Tw802 and Tw807 (Fig. 1). In contrast, none of the structural genes of enterocin A, P, L50A, Q, Bact 96, 31, 1071 A/B and HirJM 79 could be amplified.

Molecular methodologies are important in antagonism studies because they allow the identification of bacteriocin codifying genes and sequences of genes that codify antimicrobial metabolites. Genetic sequencing with the enzymatic profile of the bacteriocins in this study demonstrated the presence of bacteriocin structural genes which coded for mundticin KS, according to what was observed by other authors (Ogaki et al. 2016; Decarlo et al. 2019).

**Bacteriocin production in different culture media and incubation conditions**

For the ten strains studied, different culture media and conditions were tested to obtain the highest production of bacteriocins. In Table 4 observe the antimicrobial activity of the CFSs obtained in the different media with and without the addition of cysteine and incubating at 25, 30 and 37 °C. The higher anti-listeria activity of strains *E. mundtii* Tw56, *E. mundtii* Tw802 and *E. mundtii* Tw807 was also confirmed in the conditions tested. Bacteriocin production ranged from 200 to 819,200 AU mL⁻¹ depending on the strain, temperature, and culture media used. The lowest production of bacteriocins was obtained at 37 °C, except for that of strains *E. mundtii* Tw802 and *E. mundtii* Tw807, where the lowest production was observed at 25 °C, using LAPTgc as a culture medium. The maximum antimicrobial activity produced by the ten strains studied occurred between 25 and 30 °C, using LAPTg, except for *E. faecium* Tw6 and *E. mundtii* Tw492.
For these strains, MRSc was the best medium for bacteriocin production. Agitation (100 rpm) did not increase bacteriocin production and cysteine addition increased the antimicrobial activity of some strains (Table 4).

The results in this study indicate that the best bacteriocin production by the selected LAB occurs at temperatures between 25 and 30 °C, which do not coincide with the optimum bacterial growth temperature (37 °C). This behavior may be related to the change of the redox potential of the culture broth (Vázquez et al. 2004), and, therefore, corroborates that under conditions of low oxygen level the production of bacteriocin can be improved by LAB. Bacteriocin levels depend on the temperature, pH and nutrients, as observed in this study.

The production of bacteriocin by LAB in food depends on optimal biosynthesis but does not parallel bacterial growth, besides the interaction with components of the food matrix. (Malheiroset al. 2015; Engelhardtet al. 2018). Several studies demonstrated that LAB growth is dependent on temperature, pH and nutrient availability because LAB are microorganisms that require rich media, appropriate conditions for growth and competitive flora (Van den Berghe et al. 2006).

The results obtained in this work are in agreement with those published by other authors. Barbosa et al. (2014) determined that Lactobacillus sakei MBSa1 was grown in MRS broth at different temperatures (25, 30 and 37 °C) but the optimum temperature of bacteriocin production was 25 °C. Souza et al. (2017) determined that the production of bacteriocins by Lactococcus lactis CECT-4434 depended on the temperature of incubation, obtaining the best results in a range of 30–37 °C with or without pH control.

### Determination of the antimicrobial spectrum of CFSs produced by selected LAB

The antibacterial spectra of the antimicrobial peptides were similar and lactic cultures and Gram-negative strains were resistant to all these compounds (Table 1). E. mundtii Tw56, Tw802 and Tw807 presented the higher antimicrobial activity against L. monocytogenes Scott A. The inhibitory activity of CFSs was tested on a wide range of microorganisms and they were active against L. monocytogenes, L. innocua, Enterococcus spp. and Lactobacillus plantarum TwLb5 (Table 1). Antimicrobial activity for indicator strains showed small differences, where some strains had a lower bacteriocin production than others, which is consistent with previous studies (Aspri et al. 2017; Delcarlo et al. 2019).

### Evaluation of bacteriocinogenic strains against Listeria monocytogenes Scott A in food models

Figure 2 shows the effect of extracts of bacteriocinogenic cultures or co-inoculation of bacteriocin-producing Enterococcus against L. monocytogenes Scott A in meat gravy and ground beef incubated at 4 °C. In simulated meat gravy, the Listeria control was increased ~ 3 log CFU mL⁻¹ units with respect to day 0 (Fig. 2a). A remarkable log CFU mL⁻¹ unit decrease of the viable cell count of L. monocytogenes Scott A was observed in presence of the antilisterial extracts produced by E. mundtii (Tw56, Tw802) until disappearing completely on day 10 of treatment. In presence of the antilisterial extract produced by E. mundtii Tw807, the counts of L. monocytogenes Scott A showed no significant differences with respect to day 0.
When *L. monocytogenes* Scott A was inoculated in presence of $10^7$ (CFU mL$^{-1}$) of *E. mundtii* Tw56, Tw802 or Tw807 the counts of *Listeria* progressively decreased until disappearing completely after 15 days (Fig. 2b).

The count of *L. monocytogenes* Scott A in the control ground beef stored at 4 °C was 4.0 log CFU g$^{-1}$ and remained the same until the 15th day. In presence of the antilisterial extract produced by *E. mundtii* Tw56, no growth of *Listeria* was observed since day 5 of storage under refrigeration while a decrease occurred in the presence of the antilisterial extract produced by *E. mundtii* Tw802 until *Listeria* completely disappeared on day 7. In the ground beef with antilisterial extract produced by *E. mundtii* Tw802, no count was observed on day 10. On the other hand, it was found that the bacteriocinogenic strains were able to produce bacteriocin (1000 UA mL$^{-1}$) under these refrigeration conditions. The sensory evaluation is necessary and further studies will be carried out.

*Listeria monocytogenes* is commonly found in meats, vegetables and milk and is as an important foodborne pathogen causing various clinical syndromes. Listeriosis has been associated with consumption of a variety of foods, including soft cheeses, meat and vegetable products (Schlech and Acheson 2000) and the ability of *Listeria* to grow in a wide temperature range (1–45 °C), low pH and high salt tolerance, make it difficult to control in food (Aspri et al. 2017). Hence, the use of bacteriocins to control *Listeria* sp. in food is a promising means, whether produced in situ or added in the different food matrices.

Bacteriocins can be used for food biopreservation (addition of bacteriocin producing strains or purified or semi-purified bacteriocins) and the strategy of their use to food depends on the components present in the food matrix so that they do not affect the antimicrobial activity, due to the amphiphilic characteristic and high content of hydrophobic amino acids present in the bacteriocin, and their proteolytic degradation by enzymes.
in the food matrix (Favaro et al. 2015; O’Connor et al. 2015; Ahmad et al. 2017; Favaro and Todorov 2017).

In this work, it was observed that the antilisterial extracts of E. mundtii strains showed a similar and even better inhibitory activity against L. monocytogenes Scott A than the treatment with the respective bacteriocinogenic strains (E. mundtii Tw56, Tw802 and Tw807) in food models stored and refrigerated for 15 days. These results coincide with what was observed by Carvalho et al. (2018), who attribute this greater capacity of the antimicrobial extracts to inhibit and eliminate Listeria, because no adaptation time is required by the bacteriocinogenic strains to begin the production of antimicrobial compounds even though bacteriocin in situ production could be inhibited by food components and storage conditions (Urso et al. 2006). Other studies have assessed the bacteriocin production by LAB directly in different food matrices or the effect in the control of pathogenic bacteria when adding purified or semi-purified bacteriocins to food (Aspri et al. 2017; Lianou et al. 2017; Delcarlo et al. 2019).

In conclusion, E. mundtii Tw56, E. mundtii Tw802 and E. mundtii Tw807 were characterized as showing no presence of virulence factors, broad spectrum of antimicrobial activity, stability in a wide temperature and pH range, sensitivity to digestive proteases. In addition, they reduced the growth of L. monocytogenes Scott A in meat food models at 4 °C both digestive proteases. In addition, they reduced the growth of virulence factors, broad spectrum of antimicrobial activity, and some technological properties of bacteriocinogenic Enterococcus faecium from artisanal Tunisian fermented meat. Food Control 21:462–470. https://doi.org/10.1016/j.foodcont.2009.07.007

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Compliance with ethical standards

Conflict of interest The authors declare that they have No conflict of interest.

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