An assessment of the innocuity of *Enterococcus faecium* isolated from buffalo milk in southern Brazil.

Jéssica Pires Kurtz[1], Tiela Trapp Grassotti[1], Ana Paula Guedes Frazzon[1], Márcia Monks Jantzen[2], Amanda de Souza da Motta[1*]

[1]Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil
[2]Department of Preventive Veterinary Medicine, Universidade Federal do Rio Grande do Sul, Porto Alegre, Brazil

Abstract

Food processing is an ongoing process in the pursuit of innovation. Fermented dairy products are an example of the wide range of existing goods and cultures associated with this particular method. In this sense, starter and probiotic cultures of the same species, derived from and applied to milk, may provide greater product specificity. The objective of the present study was to identify Lactic Acid Bacteria (LAB) isolated from raw buffalo milk and assess safety indicators. By sequencing the 16S rDNA gene, LAB isolates were identified as belonging to the *Enterococcus* genus, which showed negative results for gelatin hydrolysis and hemolysin production. *E. faecium* M7AN7-1 and *E. faecium* M7AN10 were found to be susceptible to all antimicrobials tested and were selected for assays which sought to detect genes related to virulence and antimicrobial resistance. The results obtained demonstrated that *E. faecium* M7AN7-1 and *E. faecium* M7AN10 presented the necessary innocuity to continue being culture candidates for further studies concerning their functionality in food quality, whether as cultures of technological or probiotic importance.

Keywords: Lactic acid bacteria, *Enterococcus*, Innocuity, Food safety, Buffalo milk, Probiotics.

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Introduction

Buffalo milk comprises a food matrix with its own autochthonous microbiota, which still has not been thoroughly explored. However, promising results have already been observed regarding the use of this type of milk in dairy products [1]. When compared to bovine milk, in addition to presenting a lower cholesterol levels and higher protein and mineral salt contents, buffalo milk has also proven to be useful for the development of a diversity of dairy products [2]. Moreover, this category of milk presents high lactose, total solids and fat contents which, in turn, increase its yield in the elaboration of derivatives with higher nutritional values [3].

The composition of buffalo milk is characterized by a high diversity of Lactic Acid Bacteria (LAB) that confers flavor and aroma to its derivatives [4]. Upon evaluating autochthonous lactic microbiota in buffalo mozzarella cheese whey, the authors found representatives of the species *Enterococcus faecium, Enterococcus durans, Lactobacillus helveticus, Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, Lactobacillus fermentum, Lactobacillus casei* and *Leuconostoc mesenteroides* subsp. *mesenteroides*. Many of these are recognized as potential probiotic bacteria [1].

Probiotics are living microorganisms that, when ingested in adequate amounts, may confer beneficial effects on an individual's health [5]. Nevertheless, the World Health Organization establishes specific guidelines for the evaluation of the probiotic potential of bacteria. According to this guide, probiotic bacteria must have the ability to reduce the adhesion of pathogenic bacteria to the host's intestinal mucosa and produce substances with antimicrobial activity. In conjunction with these characteristics, the probiotic culture should not be resistant to antimicrobials being assessed for their safety [6].

Thus, prospecting the study of lactic acid bacteria isolates from buffalo milk becomes important for the research of potentially safe new cultures. This work aimed to identify the LAB, as well as to evaluate the innocuity properties of these cultures. These characteristics are fundamental to be researched when seeking to apply lactic acid bacteria in food.

Materials and Methods

Lactic acid bacteria and culture conditions

LAB was isolated from samples of fresh cooled buffalo milk collected from a refrigeration tank at a dairy farm located in the municipality of Cassino/RS. The isolates were kept under freezing temperatures (-20 °C) in 10% skim milk and glycerol. LAB reactivation was performed in MRS broth (Man, Rogosa and Sharpe), with incubation carried out at 30 °C for 48 h, followed by seeding of the cultures on MRS agar; plates were incubated under the same conditions. To confirm the purity of the isolates, the microorganisms were subjected to Gram staining and catalase testing.

Identification of lactic acid bacteria by the sequencing of the 16S rDNA gene

DNA extraction was performed using the thermal lysis method [7]. A Polymerase Chain Reaction (PCR) was run for 5 min at 94 °C, followed by 35 cycles of 1 min at 94 °C, 1 min and 30 sec at 53 °C and 1 min and 30 sec at 72 °C. A final extension of 4 min at 72 °C was carried out using the primer oligonucleotide with sequences F C27 3'-AGAGTTTGATCCTGGCTCAG-5' and R 530 3'-CCGCGGCTGCTGGCACGTA-5' [8]. PCR products were sequenced at the ACT Gene Molecular Analyses laboratory (at the Biotechnology Center, UFRGS, Porto Alegre, RS) using the ABI-Prism® 3500 Genetic Analyzer (Applied Biosystems) sequencer. The electropherograms obtained

were interpreted using the Chromas program, version 2.6.4 (Technelysium Pty Ltd) and compared to the database made available by the National Center for Biotechnology Information (NCBI), and their sequences were deposited on the Standard Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/).

Evaluation of the production of antimicrobial substances by lactic acid bacteria through the agar method on discs

To obtain the cell-free supernatant, a 1% inoculum of the LAB in MRS broth was incubated at 30 °C. Aliquots were then collected at 24 and 48 h of culture and centrifuged for 15 min at 10,000 rpm. Cell-free supernatants were heated for 10 min at 90 °C and pH was measured and neutralized with 0.1N NaOH. The evaluation of the antimicrobial activity was carried out through the disc-diffusion test, against the indicators *Corynebacterium fimi* NCTC 7547, *Escherichia coli* ATCC 10536, *Listeria monocytogenes* ATCC 7644, *Pseudomonas aeruginosa* ATCC 27853, *Salmonella* Enteritidis ATCC 13076 and *Staphylococcus aureus* ATCC 25923 [9]. The ability to produce antimicrobial substances was also assessed against the commercial cultures of *Lactobacillus rhamnosus* Fagron® (a probiotic) and *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus* Danisco® (a milk yeast). The plates were incubated at 30 °C for 24 h and measurement of the inhibition halos was expressed in mm. Non-pH neutralized cell-free supernatants were also used in the tests and assays were performed in duplicate.

Assessment of the safety of lactic acid bacteria

Hemolysis of erythrocytes was observed on Columbia agar plates supplemented with 5% defibrinated sheep blood; the bacterial cultures were minced and incubated for 48 h at 35 ± 1 °C [10]. The plaques were assessed for the presence of alpha hemolysis (partial), beta hemolysis (total) and gamma hemolysis (absence of hemolysis), and the test was performed in triplicate. The hydrolysis of gelatin was performed on gelatin agar [11]. Bacterial cultures were seeded in tubes containing meat extract, bacteriological peptone and 12% gelatin, and incubated for 48 h at 30 °C and then brought to a temperature of 4 °C for 30 min. This assay was performed in duplicate. *S. aureus* ATCC 25923 was used as a positive control in both tests.

Evaluation of the susceptibility of lactic acid bacteria to antimicrobials

For this analysis, the Kirby-Bauer method was employed. LAB were previously cultured on MRS agar and incubated at 30 °C for 24 h. After growth, each microorganism was transferred to a test tube containing 0.85% saline, the concentration of which was standardized according to McFarland's Scale at 0.5 (1.5×10^8 CFU mL^{-1}). Next, the cultures were seeded on Mueller Hinton agar plates using a sterile swab. Antibiotic disks were distributed over the plates and incubated at 30°C for 18-24 h. Our choice of antimicrobials took into account the recommendations of the Clinical and Laboratory Standards Institute [12]. The antimicrobials used in this trial were: ampicillin 10 µg, vancomycin 30 µg, erythromycin 15 µg, tetracycline 30 µg, ciprofloxacin 5 µg, norfloxacin 10 µg, nitrofurantoin 300 µg, chloramphenicol 30 µg, linezolid 30 µg, gentamicin 120 µg and streptomycin 300 µg. The quality controls used to evaluate the performance of antibiotic disks were *S. aureus* ATCC 25923 and *Enterococcus faecalis* ATCC 29212.

Evaluation of the presence of genes related to antimicrobial resistance and virulence factors

From the DNA samples already extracted [7], PCRs were performed to detect the following genes: ermB and msrC (for erythromycin resistance), tetL, tetM, tetS (for tetracycline resistance) and vanA, vanB, vanC1 and vanC2/3 (for vancomycin resistance). To evaluation of antimicrobial resistance genes the Table 1 shows the primer oligonucleotides used in the PCR reactions, the evaluated genes, and the sequence of each oligonucleotide and the size of the fragment generated. Table 2 shows the temperatures, number of cycles and times used in each PCR phase for all evaluated genes. The virulence genes evaluated were: ace, agg, esp, cylA and gelE. To evaluation of virulence factors the Table 3 shows the primer oligonucleotides used in the PCR reactions, the evaluated genes, and the sequence of each oligonucleotide and the size of the fragment generated. Table 4 shows the temperatures, number of cycles and times used in each PCR phase for all evaluated genes.

Table 1. *Oligonucleotide primers and PCR-generated products used to assess for the presence of antimicrobial resistance genes in lactic acid bacteria.*

Oligonucleotides	Gene	Sequence (5'- 3')	Product (bp)	Reference
erm(B) _F	ermB	GAAAAGGTACTCAACCAAATA	547	Sutcliffe et al. 1996
erm(B) _R		AGTAACGGTACTTAAATTGTTTAC		
msrC 3	msrC	AAGGAATCCTTCTCTCTCCG	343	Werner et al. 2001
msrC 4		GTAAACAAAATCGTTCCCG		
tet(L)_F	tetL	ACTCGTAATGGTGTAGTTGC	625	Frazzon et al. 2010
tet(L)_R		TGTAACTCCGATGTTTAACACG		
tet(S)_F	tetS	TGGAACGCCAGAGAGGTATT	720	Aarestrup et al. 2000
tet(S)_R		ACATAGACAAGCCGTTGACC		
van(A)_F	vanA	TAATTGAGCAGGCTGTTTCG	80	Moura et al. 2015
van(A)_R		TACTGCAGCCTGATTTGGTC		
vanB	vanB	ATGGGAAGCCGATAGTC	635	Dutka-Malen et al. 1995
vanB		GATTTCGTTCCTCGACC		
vanC1	vanC1	GGTATCAAGGAAACCTC	822	Dutka-Malen et al. 1995
vanC1		CTTCCGCCATCATAGCT		
vanC2/3	vanC2/3	CTCCTACGATTCTCTTG	439	Dutka-Malen et al., 1995
vanC2/3		CGAGCAAGACCTTTAAG		

Table 2. *Conditions used for the amplification of genes related to antimicrobial resistance in lactic acid bacteria.*

Gene	vanC2/3		Denaturation			Annealing		Extension		Final extension		Reference
	T	°C	Cycles	°C	T	°C	T	°C	T	°C	T	
ermB	3 min	93	35	93	1min	52	1min	72	1min	72	5 min	Sutcliffe et al.1996
msrC	5min	94	35	94	1min	52	1min	72	1min	72	5min	Werner et al. 2001
tetL	5min	94	35	94	1min	58	1min	72	1min	72	5min	Frazzon et al. 2010
tetM	5min	94	35	94	1min	52	1min	72	1min	72	5min	Aarestrup et al. 2000
tetS	5min	94	35	94	1min	58	1min	72	1min	72	5min	Choi and Woo, 2015
vanA	5min	94	35	94	1min	56	1min	72	1min	72	10min	Moura et al. 2015
vanB	2min	94	30	94	1min	54	1min	72	1min	72	10min	Dutka-Malen et al. 1995
vanC1	2min	94	30	94	1min	54	1min	72	1min	72	10min	Dutka-Malen et al. 1995
vanC2/3	2min	94	30	94	1min	54	1min	72	1min	72	10min	Dutka-Malen et al. 1995

Table 3. *Oligonucleotide primers and PCR-generated products used to assess for the presence of genes related to virulence factors in lactic acid bacteria.*

Oligonucleotides	Gene	Sequence (5'- 3')	Product (bp)	Reference
ace1_F	ace	AAAGTAGAATTAGATCACAC	320	Mannu et al.
ace2_R		TCTATCACATTCGGTTGCG		2003
cylA_TE17	cylA	TGGATGATAGTGATAGGAAGT	517	Eaton and Gasson (2001)
cylA_TE18		TCTACAGTAAATCTTTCGTCA		
ESP46	esp	TTACCAAGATGGTTCTGTAGGCAC	913	Shankar et al. (1999)
ESP47		CCAAGTATACTTAGCATCTTTTGG		
gelE_F	gelE	ACCCCGTATCATTGGTTT	402	Eaton and Gasson (2001)
gelE_R		ACGCATTGCTTTTCCATC		
agg TE3	agg	AAGAAAAAGAAGTAGACCAAC	1553	Eaton and Gasson (2001)
agg TE4		AAACGGCAAGACAAGTAAATA		

Table 4. *Conditions tested for the amplification of genes in relation to virulence factors in lactic acid bacteria.*

Gene	Start	Denaturation	Annealing	Extension	Final extension	Reference	agg TE4	agg TE4	agg TE4	agg TE4	agg TE4	agg TE4
	T	°C	Cycles	°C	T	°C	T	°C	T	°C	T	
ace	5min	94	35	94	1min	57	1min	72	1min	72	5min	Mannu et al. 2003
agg	5min	94	30	94	1min	62	1min	72	1min	72	10min	Eaton and Gasson, 2001
cylA	5min	94	35	94	1min	54	1min	72	1min	72	5min	Shankar et al. 1999
esp	3min	94	35	94	1min	64	1min	72	1min	72	5min	Eaton and Gasson, 2001
gelE	5min	94	35	94	1min	50	1min	72	1min	72	5min	Eaton and Gasson, 2001

Results

Identification of lactic acid bacteria by sequencing the 16S rDNA gene

The LAB M2A4, M2AN5, M7AN7, M7AN7^{-1} and M7AN10 presented coccus morphology; all five isolates were distributed into individual cells, pairs and short chains. All of the LAB mentioned above was classified as gram-positive and catalase-negative. According to the results of the 16S rDNA gene sequencing, the five LAB were identified as belonging to the genus *Enterococcus*. Isolates M2A4 and M7AN7 were identified as *Enterococcus faecalis*, while M2AN5, M7AN7^{-1} and M7AN10 were identified as *Enterococcus faecium*. The aforementioned isolates had their sequences deposited on the Standard Nucleotide BLAST (https://blast.ncbi.nlm.nih.gov/) under codes MN022498, MN022500, MN022499, MH723756 and MH723757, respectively.

Production of antimicrobial substances by lactic acid bacteria

In this test, cell-free supernatants obtained from 24 and 48 h incubation inoculum, initially presented acidic pH (4-5), which was also neutralized to pH 6. Under both conditions, we observed that, after the test of antagonism against the indicator bacteria, the halos showed similar results. All supernatant samples from both incubation periods formed halos with a mean of 8 ± 0.00 to 9 ± 1.41 mm, except *E. faecium* M7AN7^{-1}. This sample was obtained from the inoculum at 24 h of incubation, and was characterized by an acidic supernatant which formed halos measuring 10 ± 0.00 mm. The results showed that, among all bacteria evaluated for their relevance to food quality, five LAB have the potential to produce antimicrobial activity against *C. fimi* NCTC 7547. Nevertheless, other indicators were not inhibited under the experimental conditions tested.

Antimicrobial susceptibility profile

Among the five LAB evaluated, *E. faecalis* M2A4, *E. faecium* M2AN5 and *E. faecalis* M7AN7 showed an intermediate resistance profile to erythromycin, when compared with the results [12]. However, *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10 presented susceptibility to all the antimicrobials used in this experiment and were therefore selected for tests to investigate the presence of genes related to virulence and antimicrobial resistance.

Evaluation of the presence of virulence genes and antimicrobial resistance in lactic acid bacteria

Assessments for the virulence genes ace, agg, esp, cylA and gelE were carried out on *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10. However, none of these genes were detected in either LAB. With a similar test, *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10 were selected for a genetic characterization of antimicrobial resistance, to test for the presence of the genes ermB, msrC, tetL, tetM, tetS, vanA, vanB, vanC1 and vanC2/3. The msrC gene was detected in both LAB, which is responsible for conferring antimicrobial resistance to erythromycin. However, no other gene evaluated for this characteristic was detected in either LAB.

Discussion

The genus *Enterococcus* has been frequently isolated from samples of milk and its derivatives [1,13,14]. According to the results obtained in this study, all five LAB isolated from buffalo milk were identified as belonging to this genre: two isolates were identified as *E. faecalis* and three as *E. faecium*. Upon isolating bacteria of the *Enterococcus* genus from buffalo milk, were obtained a ratio that included 63.75% *E. faecalis* and 28.75% *E. faecium* [2]. Recently, with the aim of assessing for the probiotic potential of *E. faecium*, were isolated *E. faecium* (57.5%) and *E. faecalis* (15%) from samples of milk and human colostrum [15]. In research on dairy products, were isolated *E. durans* and *E. faecium* from buffalo mozzarella cheese whey, at a ratio of 35% and 15%, respectively [1]. Similarly, other work evaluated the adhesion properties of *E. faecium* and *E. faecalis* isolated from Cotija cheese, which is produced from raw milk [16]. The presence of representatives of the genus *Enterococcus* has been demonstrated as an important factor for food safety, since some strains are capable of producing enterocins which, in turn, act as biopreservatives [17,18].

In our research, LAB was evaluated for their ability to produce substances with antimicrobial potential, and demonstrated an antagonistic effect only against *C. fimi* NCTC 7547 among the tested cultures. This indicator bacterium was susceptible to all types of bacteriocins evaluated, acting as a positive control in tests that investigated the production of antimicrobial substances [19]. However, some results found, were different from those of the present study. The authors assessed four *E. faecium* isolates for their production of antimicrobial substances against *L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 6538. The halos that formed against these indicators presented sizes between 13 and 17 mm, respectively [15]. LAB is capable of producing acids, such as lactic and acetic acids, which act against pathogenic and deteriorating microorganisms [20]. This data corroborates the results obtained during our study, as an evaluation of the production of antimicrobial substances using cell-free culture supernatants with an acidic pH was also carried out. In order to counterbalance this effect, pH neutralization was likewise performed and a similar LAB response was observed. Consequently, it is possible that the antimicrobial effect could be related to the production of antimicrobial substances of another nature, such as bacteriocins, since this genus is a producer of enterocins. Enterocins are antimicrobial peptides produced by *Enterococcus* spp. which may have a broad or narrow spectrum of action [18].

In addition to the production of antimicrobial substances, other safety aspects related to the absence of virulence factors, such as the production of the enzyme gelatinase and hemolytic activity, are also important since erythrocyte hemolysis may be caused by pathogenic microorganisms [21]. The authors evaluated the hemolytic activity of *Enterococcus* spp. isolated from white cheese, and found that *E. faecalis* BP2 and *E. faecalis* PY99 showed partial hemolytic activity, while *E. faecalis* PY44 isolates showed no hemolytic activity. While some studies sought to investigate probiotic potential with the aim of highlighting benefits which could be attributed to these microorganisms, they were only able to perform limited tests

concerning food safety [22]. In some situations, genotypic tests revealed results that were not expressed in the phenotypic tests, in which the gelE gene was detected even though *E. faecium* CGLBL203 did not produce the gelatinase enzyme [23]. In the present work, a similar situation occurred, in which LAB *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10 did not express a resistance profile to erythromycin; however, in tests for the determination of antimicrobial resistance genes, the presence of the msrC gene was detected in both LAB. An important feature to be considered is the antimicrobial susceptibility profile, since microorganisms can act as reservoirs of resistance genes. *E. faecalis* plays an important role in the dissemination of resistance genes within and beyond the *Enterococcus* genus. The authors further emphasize that, while the genotypic and phenotypic profile of clinical and food isolates may be distinct, these can act as routes in the propagation of these genes [13].

Were found that, of forty *E. faecalis* isolates obtained from different cheeses produced from raw goat and bovine milk, thirty showed an intermediate resistance profile to erythromycin [13]. The data obtained by these authors are similar to those found in other work [2], who evaluated the antimicrobial susceptibility profile of *Enterococcus* spp. isolated from buffalo milk. In their study, 52.9% of *E. faecalis* isolates presented intermediate resistance to erythromycin and 65.2% of *E. faecium* isolates presented the same profile.

One of the reasons for this would be the use of antimicrobials in the treatment of infectious diseases in animals, for the purposes of growth and prophylaxis [24]. In another study whose research had the objective of assessing the susceptibility of *Enterococcus* spp. isolated from bovine rectal swabs to antimicrobials, erythromycin resistance profiles were found in 99% of the isolates. The authors attribute such results to the use of tylosin, a macrolide that may contribute to erythromycin resistance because the cellular target demonstrates action similar to both antimicrobials [25]. However, in our study, LAB were isolated from buffalo milk stored in cooling tanks at a dairy. Environmental aspects should also be taken into account as possible sources of *Enterococcus* spp. resistant to erythromycin.

Similar results were found [26], when the msrC gene was detected in all 233 clinical isolates of *E. faecium* that were evaluated, but not in the other 265 isolates of five different species of the genus *Enterococcus*. However, was investigated the frequency of this gene in *E. faecium* sourced from sewage, food, humans and animals, including isolates susceptible to macrolides. The presence of the msrC gene was detected in 121 of the 139 isolates, but 18 obtained negative results including those from human sources, sewage and poultry [27].

Conclusion

The present study identified the five LAB isolated from buffalo milk by sequencing and determining the 16S rDNA gene as belonging to the genus *Enterococcus*. The data obtained showed that no LAB presented hemolytic activity nor the production of the enzyme gelatinase. *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10 were susceptible to all assessed antimicrobials. No virulence conferring genes investigated in this study were detected, and only one gene that confers antimicrobial resistance was detected among the nine tested for that particular characteristic. Our results demonstrated that the *E. faecium* M7AN7^{-1} and *E. faecium* M7AN10 LAB were able to produce substances with antimicrobial potential and present safety characteristics that make it possible to suggest their use in the development of food products.

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***Correspondence to:**

Amanda de Souza da Motta
Microbiology Laboratory
Universidade Federal do Rio Grande do Sul
Porto Alegre, RS,
Brazil
E-mail: amanda.motta@ufrgs.br