SOMATO PUBLICATIONS

ISSN: 2641-2292

Journal of Clinical Nutrition and Food Science

Research Article

The Probiotic Properties of *Enterococcus faecium* Strains Isolated From Buffalo Milk: Food Matrix Studies

Jéssica Pires Kurtz[1], Wilian Pinto Paim[3], Márcia Monks Jantzen[2] and 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

[3]Laboratório de Virologia, Faculdade de Veterinária, Universidade Federal do Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil

*Address for Correspondence: Amanda de Souza da Motta, Microbiology Laboratory, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil, Tel: +55 51 33084111; E-mail: amanda.motta@ufrgs.br; ORCID ID: https://orcid.org/0000-0001-8637-3868

Received: 16 December 2020; Accepted: 12 March 2021; Published: 27 March 2021

Citation of this article: Kurtz, JP., Paim, WP., Jantzen, MM., Motta., AS. (2021) The Probiotic Properties of *Enterococcus faecium* Strains Isolated From Buffalo Milk: Food Matrix Studies. J Clin Nutr Food Sci, 4(1): 17-29.

Abstract

Buffalo milk has been increasingly explored in the production of dairy foods. Given the diversity of lactic acid bacteria present in this raw material, the functional potential of this type of milk should be studied. This study aims to assess the probiotic potential of two strains of *Enterococcus faecium* isolated from buffalo milk. We evaluated *E. faecium* M7AN7-1 and *E. faecium* M7AN10 for sustained cell viability under different conditions of conservation. We also assessed adhesion and cell viability in a simulated gastrointestinal transit test. *E. faecium* M7AN10 was selected for microencapsulation in sodium alginate and testing in the food matrix. The isolates maintained cell viability after refrigeration, freezing, and freeze-drying. *E. faecium* M7AN10 showed higher concentrations of viable cells than *E. faecium* M7AN7-1 after 180 min of contact with simulated gastric fluid, reaching 7.19 ± 0.59 Log_{10} CFU mL^{-1}. We also observed sustained cell viability after exposure to simulated intestinal fluid. After encapsulation, *E. faecium* M7AN10 was tested in the food matrix of UHT milk. Cell count viability was maintained and its release was sustained in this medium for 30 days. The results of the *in vitro* assessment demonstrated that *E. faecium* M7AN10 remained functionally active under these experimental conditions. Similarly, they showed that it is a bacterium capable of sustaining viability in this type of food and after transit in a simulated

gastrointestinal tract. Based on the data, we suggest this isolate may be a probiotic bacterial candidate for *in vivo* behavioral assessment.

Keywords: lactic acid bacteria; *Enterococcus*; buffalo milk; microencapsulation; probiotics

Intorduction

Buffalo milk possesses a food matrix with high levels of protein, lipid, lactose and some minerals. It also has a higher nutritional content than bovine milk, particularly protein and total solids levels. Gradually, buffalo milk has been gaining ground in the development of new food products. However, little is known about the lactic acid microbiota and the functional properties of this raw material [1,2].

Evaluating the probiotic characteristics of lactic acid bacteria (LAB) involves multiple steps and takes time. One study evaluated the properties of four lactic acid bacteria that were candidates for probiotics. *Lactobacillus casei* SJRP35, *Leuconostoc citreum* SJRP44, *Lactobacillus delbrueckii* subsp. *bulgaricus* SJRP57 and *Leuconostoc mesenteroides* subsp. *mesenteroides* SJRP58. The strains survived at different pH values and bile salt concentration, and present auto-aggregation and co-aggregation properties. The strain SJRP57 was considered the best candidate as probiotic cultures for further in vivo studies because did not show the presence of any genes encoding virulence factors [3]. *Lactobacillus rhamnosus* LB 1.5 and *Lactobacillus paracasei* LB 6.4 were buffalo milk isolates and researchers have recognized their probiotic potential and studied them in food tests [2].

The advancement of technologies seeking the better use and functional performance of lactic bacteria in products has given rise to the microencapsulation process [4]. The microencapsulation of probiotic bacteria has been described in the literature and has shown promising results. A research encapsulated

ST16Pa in sodium alginate at concentrations of 2, 3, and 4%, using the extrusion method. They evaluated the exposure of free and encapsulated cultures under simulated gastric conditions. The authors found that alginate protected the bacterial culture, regardless of the concentration used. Moreover, they measured the decrease in the viability of encapsulated microorganisms at 1.2 logs, while the free culture had its count reduced by 5 logs [5]. Similarly, were encapsulated *L. paracasei* L26, *L. casei*-01, *L. acidophilus* Ki and *B. animalis* $BB^{-1}2$ in 2% alginate and stored the microcapsules at temperatures of 21, 4, -20 and -80 ºC for six months. The results demonstrated that the loss of viability was lower in microcapsules stored at freezing temperatures [6]. According to these studies, the use of probiotics in dairy products is an important alternative for the promotion of new functional foods. Research concerning indigenous microorganisms is relevant since milk contains microbiota with many potential applications.

Material and Methods

Microorganisms and Cultivation Conditions

Previous to this study, we had already isolated the LAB from buffalo milk and identified them as *Enterococcus faecium* M7AN7-1 and *Enterococcus faecium* M7AN10. Their sequences were deposited in the *Standard Nucleotide BLAST* (https://blast.ncbi.nlm.nih. gov/) under the codes MH723756 and MH723757, respectively. These bacteria were tested at a freezing temperature of -20°C, in skim milk and 10% glycerol. The LAB were reactivated using an MRS (Man, Rogosa and Sharpe) broth culture and incubated at 35ºC for 48 h. After, the cultures on the MRS agar medium were depleted and incubated at 35ºC for 48 h to evaluate their purity. A commercial, probiotic culture of *Lactobacillus rhamnosus* (Fagron®) was used as a control.

Evaluation of the stability of lactic acid bacteria after refrigeration, freezing, and freeze-drying

For refrigeration, a sterile solution of distilled water and 10% skimmed-milk powder was prepared. To test freezing and freeze-drying, a sterile solution of distilled water and 20% skimmed-milk powder was used. The bacteria were inoculated in microtubes in these respective solutions, starting with an initial inoculum of 8.00 Log_{10} CFU mL^{-1}. Microtubes were refrigerated for 30 days or frozen for 90 days, a period during which

the cultures were thawed and a viable cell count was carried out monthly [7]. The microtubes containing lyophilized cultures were stored in a freezer for six months, and cell viability was evaluated monthly. After these treatments, testing for viable bacteria cells was performed using plates incubated at 35 ± 1ºC for 24 h. Tests were done in duplicate and results were expressed in Log_{10} CFU mL^{-1} [8].

Assessment of the tolerance of lactic acid bacteria to bile salts

Solutions containing MRS broth and bile salts were prepared in concentrations of 0.1%, 0.3%, 0.5%, and 1%. Afterward, 1000 µL of these solutions were transferred to microtubes, and 100 µL of bacterial inoculum was added. These were incubated at 35 ± 1ºC for 4 h. The control treatment used MRS broth with no added bile salts. Viable cells were counted at the beginning of the test after the 4 h period, on plates incubated at 35 ± 1ºC for 24 h. Tests were done in triplicate [9].

Evaluation of the tolerance of lactic acid bacteria to simulated gastric fluid

The test to assess the tolerance of lactic acid bacteria to simulated gastric fluid (SGF) was carried out, with some modifications [10]. This fluid was prepared using a 0.5 % NaCl solution and 0.3 g mL -1 of pepsin, with the pH adjusted to 2 with 0.1 N HCl. In microtubes, 300 µL of a 0.5% NaCl solution with 200 µL of bacterial inoculum were added. Then 500 µL of SGF were placed in these same wells. The control group was tested with 500 µL of a 0.5% NaCl solution instead of SGF. The microtubes were incubated at 35 ± 1ºC for 180 min. Aliquots of 100 µL were sampled at the beginning of the test and at 60, 90, and 180 min, to obtain viable cell counts at different times. The plates were incubated at 35 ± 1ºC for 24 h and the assay was performed in triplicate.

Assessment of the tolerance of lactic acid bacteria to simulated intestinal fluid

The simulated intestinal fluid (SIF) was a solution containing 0.5% NaCl and 0.1 g mL^{-1} of pancreatin. The pH was adjusted to 8 with 0.1 N NaOH. We added 300 µL of a 0.5% NaCl solution and 200 µL of the bacterial inoculum in microtubes. Afterward, 500 µL of SIF was added. The control group was tested with 500 µL of a 0.5% NaCl solution instead of SIF, and the microtubes were incubated at 35 ± 1ºC for 240 min. A viable cell count was performed at the beginning of the test, and after 60, 90, 180, and 240 min, using plates incubated at 35 ± 1ºC for 24 h. The experiment was realized in triplicate with some modifications [10].

Assessment of the adhesive properties of lactic acid bacteria

We tested LAB adhesion to Vero cells (the renal cells of the African green monkey *Cercopithecus aethiops*) that were kept in Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum, 10,000 U mL^{-1} of penicillin, and 10,000 U ml^{-1} streptomycin. They were incubated at 37°C and in an atmosphere of 5% CO_2. When the Vero cells reached confluence, they were added to a 96-well microplate for cell cultivation, at a concentration of 2 x 10^5 cells, and incubated under the same conditions mentioned above. Afterward, the MEM was removed from the wells and they were washed with 200 µL PBS buffer, pH 7.2. LAB cultures were inoculated in MRS broth for 24 h at 35 ± 1ºC. Afterwards, these cultures were centrifuged at 4000 rpm, at a temperature of 4ºC for 10 min. The supernatant was discarded and the precipitate was washed twice with PBS buffer and resuspended in antibiotic-free MEM. 100 µL from each culture were inoculated in wells containing Vero cells and the microplate was incubated at 37ºC for 4 h. After this period, the microplate was taken out from the oven and the bacterial suspension was removed. The wells were washed three times with 200 µL PBS buffer to remove non-adherent bacterial cells. Then, adhesion was interrupted by adding 200 µL of 0.5% Triton X100. The bacterial cell count was performed by incubating the plates at 35 ± 1ºC for 24 h. The percentage of adhered bacterial cells to Vero cells was calculated based on the relationship between the number of adhered bacteria and the number added at the beginning of the test. This test was carried out in triplicate [11-13].

Microencapsulation of the lactic acid bacteria

For this procedure, 5 mL of the bacterial inoculum were centrifuged at 2000 rpm, for 10 min at 4ºC. Afterward, the cells were washed twice with 0.1% sterile peptone water and resuspended in 5 mL of the same solution to standardize the

initial inoculum. A solution with a volume of 25 mL was prepared with 2% sodium alginate in distilled water. It was added to the cells that were previously resuspended. This content was vortexed and transferred to a sterile syringe equipped with a 0.45 mm diameter needle. The liquid was ejected into a sterile solution with 100 ml of 0.05M $CaCl_2$ supplemented with 0.1% Tween 80. The solution with the microcapsules was centrifuged at 350 rpm, at 4ºC for 10 min and washed with 0.1% sterile peptone water, and filtered through Grade 1 Whatman filter paper. The microcapsules were transferred to a flask containing 100 mL of 0.1% sterile peptone water to further test encapsulation efficiency. This test was carried out according to the methodology proposed [5].

Evaluation of the efficiency of encapsulated lactic acid bacteria

We added 1 g of sodium alginate microcapsules to 9 mL of a 2% sodium citrate solution. This content was centrifuged at 300 rpm, at a temperature of 17ºC for 10 min to release the microbial culture. After that, serial dilution was performed to determine CFU g^{-1}. Encapsulation efficiency was expressed in percentages using the following formula: Efficiency = YZ / X x100, where X represents the initial microorganism count before encapsulation; Y the count of encapsulated microorganisms and Z the count of microorganisms in the external fluid around the capsules (i.e. in the 0.05M $CaCl_2$ solution) [6]. This measure was calculated according authors [14].

Evaluation of the sustained activity of encapsulated cultures in the food matrix

This experiment was carried out in test tubes with 1 g of microcapsules and 9 mL of separate samples of whole UHT cow milk, MRS broth, and a commercial, chocolate-flavored UHT milk drink. Test tubes were kept in refrigerated storage (7°C). The release of the LAB microbial cultures was evaluated when the microcapsules were added to these liquids and after 7, 15, 20, and 30 days of the test. Each tube was vortexed for five seconds for homogenization, and then serial dilution was performed, along with pH measurement. The plates were incubated at 35 ± 1ºC for 48 h. This test was carried out in triplicate.

Statistical analysis

Variation in the averages of LAB growth in log CFU/mL^{-1} was statistically assessed using the analysis of variance (ANOVA) of repeated measures, generalized linear models, and SPSS version 18 software (SPSS Inc., Somers, NY, USA). When a significant difference was found, the Bonferroni multiple comparison test was used, with a significance level of $p \leq 0.05$.

Results Evaluation of the stability of lactic acid bacteria after refrigeration, freezing, and freeze-drying

We tested the tolerance of *E. faecium* M7AN7-1, *E. faecium* M7AN10, and *L. rhamnosus* Fagron® (a commercial probiotic bacteria used as a positive control) to refrigeration (6.6 ± 1.5 ºC) for 30 days. Weekly cell counts showed that the three LAB began and completed tests with numbers higher than 8 Log_{10} CFU mL^{-1}. *L. rhamnosus* demonstrated the highest viability, followed by *E. faecium* M7AN7-1 and then *E. faecium* M7AN10 (Table 1). Using the Bonferroni multiple comparison test, we observed a significant difference ($p \leq 0.05$) between the variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1}, and those of *E. faecium* M7AN10 and *E. faecium* M7AN7-1. This variation depended on the time when the viable cell count was done. However, since *E. faecium* M7AN10 and *E. faecium* M7AN7-1 presented results higher than 6 Log_{10} CFU mL^{-1} (Table 1), they maintained sufficient viability to be considered stable cultures under refrigeration.

The cell viability of the *E. faecium* M7AN7-1, *E. faecium* M7AN10, and *L. rhamnosus* strains in frozen 20% powdered skim milk was tested over 90 days. The monthly viable cell counts demonstrated that, at the beginning of the experiment, all three LAB showed cell viability higher than 8 Log_{10} CFU mL^{-1}. These levels remained consistent after 90 days of being frozen (Table 2), with the *E. faecium* M7AN7-1 strain presenting the highest numbers of viable cells, followed by *L. rhamnosus* and *E. faecium* M7AN10 respectively. Variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1} was statistically different ($p \leq 0.05$) from that of *E. faecium* M7AN10 in all cell counts, except after 90 days of being frozen. Despite these variations, *E. faecium* M7AN10 maintained cell viability and presented results greater than 6 Log_{10} CFU mL^{-1}. Monthly cell viability counts were performed on the freeze-dried *E. faecium* M7AN7-1, *E. faecium* M7AN10, and *L. rhamnosus* strains. The initial count was as follows: *E. faecium* M7AN7-1 9.14

Table 1: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* (FAGRON) in refrigerated 10% powdered skim milk at 7, 14, 21 and 30 days. Values are expressed as Log10 CFU mL-1.

LAB	Before refrigeration	7th day	14th day	21st day	30th day
E. faecium M7AN7-1	8.53 ± 0.13^{abA}	8.70 ± 0.04^{aB}	8.59 ± 0.02^{aAB}	8.42 ± 0.20^{abA}	8.59 ± 0.01^{aA}
E. faecium M7AN10	8.46 ± 0.03^{aA}	8.48 ± 0.02^{bB}	8.44 ± 0.01^{bA}	8.30 ± 0.07^{aC}	8.46 ± 0.09^{aAB}
L. rhamnosus	8.60 ± 0.05^{bA}	8.70 ± 0.01^{aA}	8.72 ± 0.04^{cA}	8.67 ± 0.09^{bA}	8.81 ± 0.04^{bB}

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column and identical uppercase letters in the same row indicate no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test.

Table 2: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* (FAGRON) in frozen 10% powdered skim milk, at 30, 60 and 90 days. Values are expressed as Log_{10} CFU mL^{-1}.

LAB	Before freezing	30th day	60th day	90th day
E. faecium M7AN7-1	8.88 ± 0.07^{aA}	8.54 ± 0.09^{aB}	8.58 ± 0.01^{aB}	8.50 ± 0.05^{aB}
E. faecium M7AN10	8.41 ± 0.01^{bA}	8.34 ± 0.01^{bB}	8.41 ± 0.05^{bAB}	8.16 ± 0.05^{bC}
L. rhamnosus	8.83 ± 0.04^{aA}	8.53 ± 0.08^{aB}	8.57 ± 0.02^{aB}	8.23 ± 0.27^{abB}

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column and identical uppercase letters in the same row indicate no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test.

± 0.07, *E. faecium* M7AN10 8.50 ± 0.10, and *L. rhamnosus* 9.16 ± 0.01. Even though there was a small decrease after lyophilization, the LAB maintained cell counts greater than 8 Log_{10} CFU mL^{-1} (Table 3). *L. rhamnosus* showed the highest concentrations of viable cells 180 days after the lyophilization process, indicating the greatest tolerance to the process. This level of tolerance was followed by that of *E. faecium* M7AN7-1 and then *E. faecium* M7AN10. After using the Bonferroni multiple comparison test, we verified that the variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1} differed significantly ($p \leq 0.05$) from those of *E. faecium* M7AN10 in all test periods, always reaching more than 6 Log_{10} CFU mL^{-1}.

Tolerance to bile salts

In this test, the LAB presented values above 7 Log_{10} CFU mL^{-1} when exposed to 0.1% bile salts. However, only the *E. faecium* M7AN7-1 and *E. faecium* M7AN10 strains remained viable after contact with 0.3 and 0.5% bile salts for 4 h, demonstrating cell counts greater than 6 Log_{10} CFU mL^{-1} (Table 4). *E. faecium* M7AN10 remained stable after being exposed to 1% bile salts and reached cell counts of 4 Log_{10} CFU mL^{-1}, unlike *L. rhamnosus* that did not tolerate exposure to 0.3, 0.5, and 1% bile salts. In the control treatment (with no added bile salts), the cell counts were greater than 8 Log_{10} CFU mL^{-1} (Table 4). The Bonferroni multiple comparison test showed that the variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1} differed significantly ($p \leq 0.05$) from those of *E. faecium* M7AN7-1 and *E. faecium* M7AN10. This demonstrated that both LAB showed stability under the tested conditions.

Tolerance to simulated gastric fluid

The *E. faecium* M7AN10 and *L. rhamnosus* isolates maintained their viability throughout the test period, reaching cell counts above 7 Log_{10} CFU mL^{-1} and 8 Log_{10} CFU mL^{-1}, respectively. However, *E. faecium* M7AN7-1 lost its viability after 90 minutes of

Table 3: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* (FAGRON) in freeze-dried 20% powdered skim milk at 30, 60, 90, 120, 150 and 180 days. Values are expressed as Log_{10} CFU mL^{-1}.

LAB	Freeze-drying (0 h)	30th day	60th day	90th day	120th day	150th day	180th day
E. faecium M7AN7-1	8.65 ± 0.05^{aE}	8.71 ± 0.02^{aA}	8.72 ± 0.02^{aB}	8.89 ± 0.03^{aC}	8.75 ± 0.09^{aD}	8.86 ± 0.01^{aE}	9.02 ± 0.02^{aF}
E. faecium M7AN10	8.49 ± 0.09^{aBCDEF}	8.53 ± 0.08^{bA}	8.47 ± 0.10^{bB}	8.53 ± 0.01^{bC}	8.43 ± 0.01^{bD}	8.49 ± 0.01^{bE}	8.45 ± 0.01^{bF}
L. rhamnosus	9.01 ± 0.01^{bABE}	9.01 ± 0.20^{aA}	9.06 ± 0.11^{cB}	9.11 ± 0.01^{cC}	9.10 ± 0.05^{cD}	8.94 ± 0.21^{aE}	9.12 ± 0.05^{cF}

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column and identical uppercase letters in the same row indicate no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test.

Table 4: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus,* in 0.1%, 0.3%, 0.5% and 1% of bile salts for 4 h. Values are expressed as Log_{10} CFU mL^{-1}.

LAB		After 4 h				
	0 h	Control	0.10%	0.30%	0.50%	1%
E. faecium M7AN7-1	7.40 ± 0.03^{aB}	8.52 ± 0.08^{a+}	7.30 ± 0.05^{bA-}	6.33 ± 0.10^{aC*}	6.51 ± 0.05$^{aD/}$	0
E. faecium M7AN10	6.99 ± 0.09^{bA}	9.02 ± 0.21^{b+}	7.02 ± 0.04^{bA-}	6.98 ± 0.06^{bA-}	6.97 ± 0.03^{bA-}	4.42 ± 0.21^{B*}
L. rhamnosus	7.67 ± 0.17^{cA}	8.26 ± 0.01^{c+}	7.58 ± 0.05^{cA-}	0	0	0

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column and identical uppercase letters in the same row indicate that no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test. For the control treatment: identical symbols in the same row indicate no statistical difference ($p > 0.05$).

contact with SGF, when results fell below 6 Log_{10} CFU mL^{-1} (Table 5). Regarding the control treatment (with no added SGF), all LAB strains maintained viability and presented cell counts greater than 6 Log_{10} CFU mL^{-1}. Using the Bonferroni multiple comparison test, we verified that the variation in the averages *L. rhamnosus* growth in log CFU/mL differed significantly ($p \leq 0.05$) from those of *E. faecium* M7AN7-1 and *E. faecium* M7AN10. However, this depended on the time when viable cell counts were performed. The results of this part of the experiment demonstrated that *E. faecium* M7AN7-1 did not present the expected viable cell counts after 180 min of contact with SGF.

Tolerance to simulated intestinal fluid

The LAB remained viable after exposure and reached cell counts above 8 Log_{10} CFU mL^{-1}. The *E. faecium* M7AN7-1 isolate showed the highest concentrations of viable cells after 240 min of contact with the SIF, followed by *E. faecium* M7AN10 and *L. rhamnosus*. The variation in the averages of LAB growth in log CFU mL^{-1} depended on the evaluation time (Table 6). The Bonferroni multiple comparison test demonstrated that the variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1} differed significantly ($p \leq 0.05$) from those of *E. faecium* M7AN7-1 and *E. faecium* M7AN10. However, both of these LAB presented the viable cell counts that suggested stability under the experimental conditions (i.e. a cell count equal to or greater than 6 Log_{10} CFU mL^{-1}). In the control treatment, variations in the averages of growth in log CFU mL^{-1} were independent of the evaluation time, and we found a statistically significant difference ($p \leq 0.05$) between the LAB.

Table 5: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* in simulated gastric fluid (SGF) at 60, 90 and 180 min. Values are expressed as Log_{10} CFU mL^{-1}.

LAB	0 h	60 min		90 min		180 min	
		Control	SGF	Control	SGF	Control	SGF
E. faecium M7AN7-1	$7.96 \pm 0.30^{aA+}$	$8.18 \pm 0.05^{a+}$	7.18 ± 0.29^{aA}	$8.17 \pm 0.02^{a+}$	5.82 ± 0.48^{aB}	$8.04 \pm 0.16^{a+}$	3.32 ± 0.49^{aC}
E. faecium M7AN10	$7.90 \pm 0.20^{aA+}$	$7.20 \pm 1.12^{a+}$	7.51 ± 0.18^{aB}	$6.08 \pm 0.96^{b-}$	7.62 ± 0.35^{bAB}	$6.87 \pm 1.14^{a+-}$	$7.19 \pm 0.5^{9b}B$
L. rhamnosus	$8.15 \pm 0.04^{aA+-}$	$8.09 \pm 0.03^{a+-}$	8.18 ± 0.09^{bA}	$8.20 \pm 0.04^{a+}$	7.70 ± 0.83^{bA}	$8.15 \pm 0.02^{a-}$	8.12 ± 0.03^{cA}

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column and identical uppercase letters in the same row indicate that no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test.

Table 6: Survival of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* in simulated intestinal fluid (SIF) at 60, 90, 180 and 240 min. Values are expressed as Log_{10} CFU mL^{-1}.

LAB	0 h	60 min		90 min		180 min		240 min	
		Control	SIF	Control	SIF	Control	SIF	Control	SIF
E. faecium M7AN7-1	$8.31 \pm 0.03^{Aa+}$	$8.25 \pm 0.09^{a-}$	8.40 ± 0.02^{aB}	$8.20 \pm 0.07^{a*}$	8.44 ± 0.00^{aB}	$8.24 \pm 0.04^{a/}$	8.58 ± 0.00^{aC}	$8.24 \pm 0.04^{a!}$	8.60 ± 0.03^{aC}
E. faecium M7AN10	$7.98 \pm 0.13^{bA+}$	$7.97 \pm 0.02^{b-}$	8.18 ± 0.01^{bB}	$8.03 \pm 0.01^{b*}$	8.42 ± 0.03^{aC}	$7.93 \pm 0.11^{b/}$	8.32 ± 0.02^{bD}	$7.95 \pm 0.06^{b!}$	8.28 ± 0.04^{bD}
L. rhamnosus	$8.13 \pm 0.15^{cA+}$	$7.99 \pm 0.05^{c-}$	8.22 ± 0.09^{bA}	$8.23 \pm 0.02^{c*}$	8.24 ± 0.04^{bA}	$8.11 \pm 0.05^{c/}$	8.09 ± 0.09^{cA}	$8.14 \pm 0.08^{c!}$	8.14 ± 0.13^{bA}

Values are expressed as mean ± standard deviation. Identical lowercase letters in the same column indicate no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test. For the control treatment: identical symbols in the same row indicate no statistical difference ($p > 0.05$).

Adhesive properties of lactic acid bacteria

All three LAB started the experiment with cell counts greater than 8 Log_{10} CFU mL^{-1}. However, only 5 Log_{10} CFU mL^{-1} of the *L. rhamnosus* isolate were able to adhere to Vero cells during testing. The *E. faecium* M7AN7-1 and *E. faecium* M7AN10 strains showed more satisfactory adhesion to Vero cells, exhibiting a value of 6 Log_{10} CFU mL^{-1}. This demonstrated a superior adhesion capacity over that of *L. rhamnosus* (Figure 1). In terms of percentages of adherence, *E. faecium* M7AN7-1 presented 1.23% adhesion while *E. faecium* M7AN10 showed 0.33% adhesion. At the beginning of the experiment, the variation in the averages of *L. rhamnosus* growth in log CFU mL^{-1} differed significantly ($p \leq 0.05$) from that of *E. faecium* M7AN7-1. However, after 4 h of contact with Vero cells, the variation in the averages of growth of all the LAB in log CFU mL^{-1} was statistically different ($p \leq 0.05$). Results showed that the *E. faecium* M7AN7-1 and *E. faecium* M7AN10 isolates presented viable cell counts that are suggestive of adhesive potential (i.e. equal to or greater than 6 Log_{10} CFU mL^{-1}).

The efficiency of microencapsulating *Enterococcus faecium* M7AN10

After the microencapsulation of the *E. faecium* M7AN10 and *L. rhamnosus* (control) isolates in 2% alginate, we tested the efficiency of the process. *E. faecium* M7AN10 presented 80.74% efficiency while *L. rhamnosus* showed 87.32% efficiency. Before undergoing microencapsulation, both LAB had cell counts above 8 Log_{10} CFU mL^{-1}. However, there was a reduction in the cell viability of both after this procedure: *L. rhamnosus* reached 7 Log_{10} CFU mL^{-1} and *E. faecium* M7AN10 reached 6 Log_{10} CFU mL^{-1} for *E. faecium* (Figure 2).

Evaluation of encapsulated lactic acid bacteria in a food matrix

Microencapsulated LAB cultures in UHT whole milk and MRS broth were monitored. The release of bacterial cultures from the

microcapsules occurred differently way for each of the LAB. The release of the *L. rhamnosus* bacteria occurred from the 15th day in UHT whole milk and the 7th day in MRS broth. At the end of testing, only 5 Log_{10} CFU mL^{-1} had been released in both media. In contrast, *E. faecium* M7AN10 bacteria were released from the microcapsules at zero time, both in the UHT whole milk and the MRS broth. One aspect that could explain this early result is the ability of viable *E. faecium* M7AN10 cells to adhere to the capsules. After 30 days, the viability of this strain was measured at 8 Log_{10} CFU mL^{-1} in milk and 6 Log_{10} CFU mL^{-1} in MRS broth (Figure 3). The variation in the averages of the *E. faecium* M7AN10 growth in log CFU mL^{-1} differed significantly ($p \leq 0.05$) from that of *L. rhamnosus*, whether in relation to the UHT whole milk or the MRS broth and regardless of the moment of the viable cell count.

The 2% alginate microcapsules containing *E. faecium* M7AN10 (6.51 ± 0.04 Log_{10} CFU mL^{-1}) were tested in a commercial UHT chocolate-flavored milk drink, and its release in this culture was assessed for 30 days. At the beginning of the test (time zero), 3.97 ± 0.65 Log_{10} CFU mL^{-1} of this bacterium had already been released in the UHT chocolate milk drink; however, on the 30th day, the viable cell count in this food matrix increased to 7.37 ± 0.45 Log_{10} CFU mL^{-1} (Figure 4). The pH was also measured during this experiment. At time zero the pH was 6, but from the 7th to the 30th day, it fell to 5.

Discussion

The *Enterococcus* genus has often been isolated from samples of milk and dairy products [15-18]. Some research has aimed to assess and highlight the potential probiotic benefits of these microorganisms. As such, it is important to assess the stability of these isolates under conditions that mimic those of dairy product conservation and processing [19].

The ability of LAB to tolerate low temperatures is an essential question to address since the vast majority of products containing probiotic cultures are dairy derivatives that require storage under cold conditions [20-24]. The results of this study demonstrated that the LAB maintained cell viability after the experiments of refrigeration, freezing, and freeze-drying. These data corroborate those reported by other authors [7]. In their experiment, they assessed the ability of *Bifidobacterium* to maintain viability under refrigerated and frozen conditions. Viable cell counts measured between 8.9 to 9.7 Log_{10} CFU mL^{-1} after six months of testing at a temperature of -20ºC. The authors also reported viability after four weeks of testing at a temperature of 4 ºC, and viable cell counts ranged from 8.2 to 9.1 Log_{10} CFU mL^{1}. The lyophilization of LAB is an important process, as many industries market their goods in this way. Was evaluated the tolerance of encapsulated *L.*

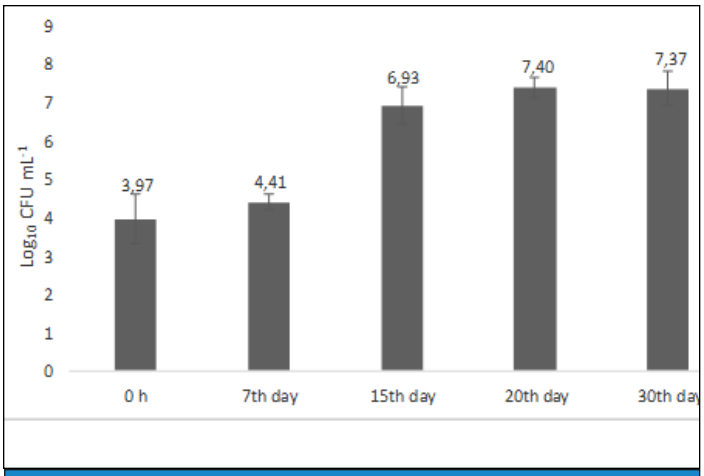

Figure 1: Viable cell count of *E. faecium* M7AN7-1, *E. faecium* M7AN10 and *L. rhamnosus* (control) before and after 4 h of contact with the Vero cells. Values are expressed as mean ± standard deviation. Identical lowercase letters in the same moment, but for different LAB, indicate no statistical difference ($p > 0.05$) after using the Bonferroni multiple comparison test.

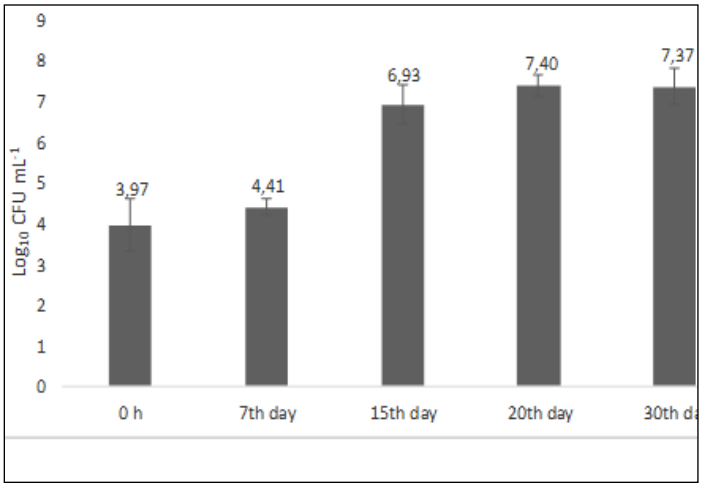

Figure 2: Viable cell count of *L. rhamnosus* and E. faecium M7AN10 before and after microencapsulation. Values are expressed as mean ± standard deviation.

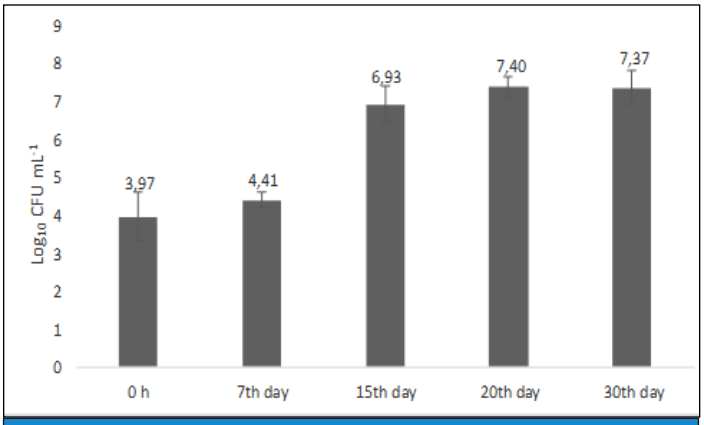

Figure 3: Viable cell count of *E. faecium* M7AN10 and *L. rhamnosus* (control) released from the microcapsules into UHT whole milk and MRS broth at 7, 15, 20 and 30 days. Values are expressed as mean ± standard deviation. Identical lowercase letters, at the same times and for the same treatments, but for different LAB, indicate no statistical difference (p > 0.05) after using the Bonferroni multiple comparison test.

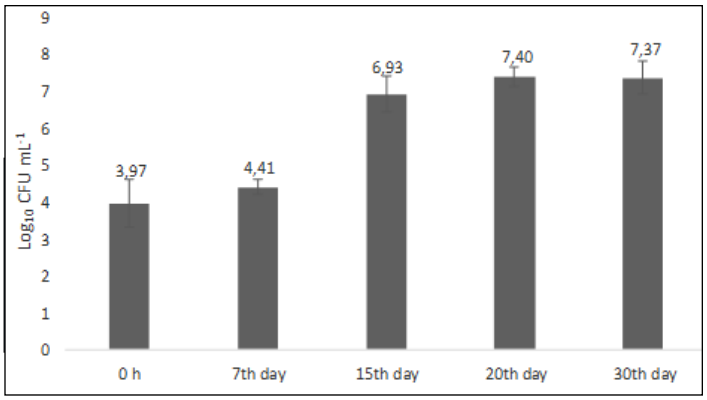

Figure 4: Viable cell count of *E. faecium* M7AN10 released from microcapsules into the UHT flavored milk drink at 7, 15, 20 and 30 days. Values are expressed as mean ± standard deviation.

casei DSPV 318T and *L. plantarum* DSPV 354T to lyophilization and its stability at a refrigerated storage temperature (4ºC). The authors found that the microorganisms maintained viability after lyophilization (regardless of the type of cryoprotectant) and after the freeze-dried capsules were placed in refrigerated storage for 84 days [25].

The cell viability of probiotic cultures should not only be studied within the context of food matrices. Transit through the gastrointestinal tract should also be considered. To offer benefit, probiotics must be able to tolerate the effects of bile salts, gastric fluid, and intestinal fluid [26]. For this reason, studies that evaluate probiotic potential should take into account the capacity of microorganisms to face these adverse conditions. *E. durans* LAB18s was isolated from Minas fresh cheese and tested it against different concentrations of bile salts (0.1, 0.25, 0.50, 1.0, and 1.5%) for 4 h. This bacterium was able to tolerate all concentrations during the experiment, and present counts greater than 8 Log $_{10}$ CFU mL^{-1} [27]. Our results showed reduced viability since *E. faecium* M7AN7-1 and *E. faecium* M7AN10 registered counts below 7 Log $_{10}$ CFU mL $^{-1}$ after 4 h of contact with 0.5% bile salts. Although there was an increase in viability after exposure to 0.1 and 0.3% bile salts, only *E. faecium* M7AN10 registered viable cell counts (4.42 ± 0.21 Log $_{10}$ CFU mL^{-1}) after contact with 1% bile salts. *E. faecium* was isolated from artisanal cheeses and tested their tolerance to 0.3% bile salts. The authors found that 24 of 103 isolates were able to tolerate that concentration of bile salts for 240 min [28]. However, other authors demonstrated that *E. faecalis* UGRA10 lost its viability after exposure to 0.3% bile salts [29].

To evaluate tolerance to simulated gastric fluid (pH 2), were isolated four strains of *E. faecium* from human milk and found that after 1 h of contact with the simulated solution, cell counts lowered from 8 Log$_{10}$ CFU mL^{-1} to approximately 2 to 3 Log$_{10}$ CFU mL^{-1}. After 3 h, counts were <1. Our results varied among the LAB. *E. faecium* M7AN7-1 presented a cell count of approximately 3 Log$_{10}$ CFU mL^{-1}, while *E. faecium* M7AN10 presented results above 7 Log $_{10}$ CFU mL $^{-1}$ after 3 h [30]. However, were published results that demonstrated the high viability of *E. faecium* SJRP20 and SJRP65. The authors performed a consecutive evaluation by simulating the gastric phase (pH 2.5), followed by a simulated enteric phase (pH 8). After the first phase, the viability of both strains was greater than 9 Log $_{10}$ CFU mL^{-1}. After the second phase, the cell count reached 8 Log $_{10}$ CFU mL^{-1}, approximately [31]. Similar results were obtained by other authors, that used a simulated gastric and intestinal transit test to assess *L. paracasei*. They reported cell count viability of approximately 8 Log $_{10}$ CFU mL $^{-1}$ and 7 Log $_{10}$ CFU mL $^{-1}$ for each of the phases [32].

However, some researchers have shown that tolerance to intestinal fluid is a more important property because some foods can act as buffers in the gastric tract and help probiotics reach the small intestine [27]. Was evaluated the viability of *E. faecium* exposed to

0.1 g mL $^{-1}$ of pancreatin at pH 8, for 240 min. They noted that 45% of the isolates showed tolerance to this adverse condition, showing survival rates ranging from 78.6 to 99.2%, depending on the strain [28]. The tolerance of *L. plantarum* BLS29 was assessed to intestinal fluid after 4 h of exposure and reported cell count viability above 8 Log_{10} CFU mL^{-1}. Maintaining viability after gastrointestinal transit is an important aspect because probiotic cultures need to colonize the intestine to fully exercise their functional potential [33].

The ability to adhere to epithelial cells is also an important property for a probiotic since this characteristic will determine its competence in colonizing the intestine. The results found in the literature are usually described as a percentage of adhesion. A research evaluated the adhesion capacity of *L. plantarum* LB95 and *L. plantarum* LB13 to HT-29 cells (human adenocarcinoma epithelial cells) and found that adhesion percentages ranged from 0.7 to 1.8%. In some situations, the ability to adhere may differ between certain types of epithelial cells, as well as between different LAB species belonging to the same genus [34]. The adhesion of *Lactobacillus* spp. to Vero and HeLa cells (human carcinoma epithelial cells) was evaluated. The authors observed that Vero cells adhered more efficiently than HeLa cells [12]. Still, on the topic of the different types of cells that have been used in research, was reported the adhesion of *Leuconostoc mesenteroides* subsp. *mesenteroides*, *E. faecium*, and six different *L. plantarum* isolates to Caco-2 cells (human colon adenocarcinoma epithelial cells). They considered high adhesion values to be between 4 and 8%, and their results showed adhesion levels ranging between 1.3 and 8% [13]. However, other work evaluated the adhesion of five *E. faecium* isolates to Caco-2 and TC7 cells (human colon adenocarcinoma epithelial cells). Three isolates presented adhesion percentages of 0.7, 0.9, and 1.4% (very low adhesion). One isolate showed 7% adhesion (moderate adhesion) while another demonstrated 21% adhesion (high adhesion) [35].

Regarding the adhesion percentages of our isolates, the *E. faecium* M7AN7-1 and *E. faecium* M7AN10 strains presented 1.23% and 0.33% adhesion, respectively. However, the results of the viable cell count showed that 6.93 Log_{10} CFU mL $^{-1}$ of *E. faecium* M7AN7-1 and 6.14 Log_{10} CFU mL $^{-1}$ of *E. faecium* M7AN10 were able to adhere to Vero cells.

For a probiotic culture to maintain its viability after gastrointestinal transit, adhere to the intestine, and consequently colonize it, microencapsulation can be used to minimize the impacts caused by the adverse conditions of the gastrointestinal tract. In addition to protecting microorganisms from damage, microencapsulation helps probiotics maintain viability during the temperature fluctuations and pH changes that are typical of food production technologies and food storage [21,36].

Some encapsulation methods have demonstrated efficiency with some microorganisms, but not with others. *E. faecium* IS-27526 and *L. plantarum* IS-10506 probiotic strains were encapsulated in 4.75% alginate, using fluidized bed technology. They then tested these microcapsules in a system that simulated the gastric tract and small intestine. Their results showed that the *L. plantarum* IS-10506 strain presented 84.5% viability, while only 15.7% of the *E. faecium* IS-27526 strain remained viable [37].

Some encapsulating materials can also enhance sustained LAB viability better than others. A research encapsulated *L. acidophilus* in separate alginate and carrageenan 2% concentrations, using an automated extrusion method. The carrageenan encapsulated the microorganism with 96% efficiency, while the alginate presented 98% efficiency [22].

Using encapsulated microorganisms in food is an option for extending viability beyond that of free-form probiotics. 3% alginate and the emulsion method were used to encapsulate *L. salivarius* CET 4063 and *L. paracasei* A13 before testing them in fermented milk. This dairy derivative was stored at 4 °C, and the cell count viability of these microorganisms was evaluated for 56 days. The encapsulated LAB remained significantly ($p \leq 0.05$) more viable than those in free form [21].

Ice cream has also been shown to be a good food matrix for encapsulated probiotics. To test increased viability, was used the emulsion process to combine 3.6% alginate, 2% starch, and 6% glucose (a cryoprotectant) and encapsulate *B. longum* CFR815j. After 15 days of storage at -20 °C, the results showed a 2.5% reduction in the viability of the encapsulated form of the probiotic. This was different from the 40% reduction in the viability of its free form [23]. Similarly, other research used separate 2% concentrations of alginate and carrageenan to encapsulate *L. acidophilus*, and

study the microcapsules in ice cream. They monitored their viability for 120 days. The results showed that this bacterium remained significantly ($p \leq 0.05$) more viable in its encapsulated form, whether coated in alginate or carrageenan, than in its free form. These data confirm that microencapsulation effectively maintains the viability of microorganisms in foods that need to be stored at low temperatures [22].

When we tested the encapsulated probiotic cultures in a UHT flavored milk drink and UHT whole milk, we noted that the microcapsules played an important role in maintaining the viability of *E. faecium* M7AN10. This efficiency was reflected in the cell counts because, during 30 days of storage in a refrigerator, they were greater than 7 and 8 Log_{10} CFU mL^{-1}, respectively. This established the necessary cell count viability for a probiotic bacterium. According to the Brazilian National Health Surveillance *E. faecium* is listed as a recognized probiotic. Our results showed that the *E. faecium* M7AN10 strain maintained the cell count viability attributed to probiotic cultures [38].

Based on the data of our *in vitro* tests, and comparisons with the information found in the literature, we suggest that the *E. faecium* M7AN10 strain may be a potential probiotic. However, *in vivo* tests must be carried out to see if it sustains probiotic characteristics and cell count viability.

Conclusion

This study explored the probiotic potential and stability of *Enterococcus faecium* isolates at different temperatures. Two LAB strains remained viable after maintenance and stability tests under different conditions. However, in tests for probiotic potential, *E. faecium* M7AN10 performed better after exposure to simulated gastric fluid.

E. faecium M7AN10 was microencapsulated in alginate, with good encapsulation efficiency. After this evaluation, *E. faecium* M7AN10 was tested in UHT milk. After 30 days in this food, the cell count viability of this bacterium was measured at values above 7 Log_{10} CFU mL^{-1}.

The results we obtained after identifying isolates of native lactic acid bacteria from this underexplored raw material highlight the importance of searching for new isolates that may show promising probiotic potential. Giving visibility to the natural functional potential of buffalo milk will promote interest in the development of naturally functional buffalo dairy products. Further research is needed to study these isolates *in vivo*.

Acknowledgment

We thank the Virology Laboratory of the Veterinary Faculty of the Federal University of Rio Grande do Sul.

Conflict of Interest

The authors declare that there is no conflict of interest

References

1. Godinho, F., Krug, M., Figueiredo, R., Müller, A., Jank, L., Tomaszewski, C, et al. (2020) Microbiological and physicochemical characteristics of buffalo milk used for dairy products in southern Brazil. Journal of Dairy Research, 87(4): 463-468.
2. Breyer, GM., Arechavaleta, NN., Siqueira, FM., Motta, AS. (2020) Characterization of Lactic Acid Bacteria in Raw Buffalo Milk: a Screening for Novel Probiotic Candidates and Their Transcriptional Response to Acid Stress. Probiotics Antimicrob Proteins, doi: 10.1007/s12602-020-09700-4.
3. Jeronymo-Ceneviva, AB., Paula, AT., Silva, LF., Todorov, SD., Franco, BDGM., Penna, ALB. (2014) Probiotic properties of lactic acid bacteria isolated from water-buffalo mozzarella cheese. Probiotics Antimicrob Proteins, 6(3-4): 141-56.
4. Krasaekoopt, W., Bhandari, B., Deeth, H. (2003) Evaluation of encapsulation techniques of probiotics for yoghurt. International Dairy Journal, 13(1): 3-13.
5. Todorov, SD., LeBlanc, JG., Franco, BDGM. (2012) Evaluation of the probiotic potential and effect of encapsulation on survival for Lactobacillus plantarum ST16Pa isolated from papaya. World J Microbiol Biotechno, 28(3): 973-984.
6. Sousa, S., Gomes, AM., Pintado, MM., Malcata, FX., Silva, JP., Sousa, JM., et al. (2012) Encapsulation of probiotic strains in plain or cysteine-supplemented alginate improves

viability at storage below freezing temperatures. Eng Life Sci, 12(4): 457-465.

7. Zacarías, MF., Binetti, A., Laco, M., Reinheimer, J., Vinderola G. (2011) Preliminary technological and potential probiotic characterization of bifidobacteria isolated from breast milk for use in dairy products. International Dairy Journal, 21(8): 548-555.
8. Miles, AA., Misra, SS., Irwin, JO. (1938) The estimation of the bactericidal power of the blood. J Hyg (Lond), 38(6): 732-749.
9. Meira, SMM., Helfer, VE., Velho, RV., Medina, LFC., Brandelli, A. (2010). Identification and resistance to biological barriers of lactic acid bacteria isolated from milk and sheep cheese. Brazilian Journal of Food Technology. 3:75-80.
10. Charteris, WP., Kelly, PM., Morelli, L., Collins, JK. (1998) Development and application of an in vitro methodology to determine the transit tolerance of potentially probiotic Lactobacillus and Bifidobacterium species in the upper human gastrointestinal tract. J Appl Microbiol, 84(5): 759-768.
11. Abbasiliasi, S., Tan, JS., Bashokouh, F., Ibrahim, TAT., Mustafa, S., Vakhshiteh, F., et al. (2017) In vitro assessment of Pediococcus acidilactici Kp10 for its potential use in the food industry. BMC Microbiology, 17(121): 2-11.
12. Mousavi, E., Makvandi, M., Teimoori, A., Ataei, A., Ghafari, S., Najafian, M., et al. (2016) In vitro adherence of Lactobacillus strains isolated from the vaginas of healthy Iranian women. J Chin Med Assoc, 79(12): 665-671.
13. Todorov, SD., Dicks, LMT. (2008) Evaluation of lactic acid bacteria from kefir, molasses and olive brine as possible probiotics based on physiological properties. Food Microbiology, 58(4): 661-670.
14. Capela, P., Hay, TKC., Shah, NP. (2007) Effect of homogenisation on bead size and survival of encapsulated probiotic bacteria. Food Research International, 40(10):1261-1269.
15. Silvetti, T., Morandi, S., Brasca, M. (2019) Does Enterococcus faecalis from Traditional Raw Milk Cheeses Serve as a Reservoir of Antibiotic Resistance and Pathogenic Traits? Foodborne Pathog Dis, 6(5): 359-367.
16. Ispirli, H., Demirbas, F., Dertli, E. (2017) Characterization of functional properties of Enterococcus spp. isolated from Turkish white cheese. LWT, 75: 358-65.
17. Silva, LF., Casella, T., Gomes, ES., Nogueira, MCL., Dea Lindner, J., Penna, ALB. (2015) Diversity of lactic acid bacteria isolated from Brazilian water buffalo mozzarella cheese. J Food Sci, 80(2): 411-417.
18. Prichula, J., Zvoboda, DA., Pereira, RI., Santestevan, NA., Medeiros, AW., Motta, AS., et al. (2013) Antimicrobial susceptibility profile and diversity of enterococcus species isolated from raw buffalo milk in southern Brazil. Revista Brasileira de Ciência Veterinária, 20(2): 104-09.
19. Todorov, SD., Perin, LM., Carneiro, BM., Rahal, P., Holzapfel ,W., Nero, LA. (2017) Safety of Lactobacillus plantarum ST8Sh and Its Bacteriocin. Probiotics Antimicrob Proteins, 9(3): 334-344.
20. Murtaza, MA., Huma, N., Shabbir, MA., Murtaza, MS., Anees-ur-Rehman, M. (2017) Survival of micro-organisms and organic acid profile of probiotic Cheddar cheese from buffalo milk during accelerated ripening. International Journal of Dairy Technology, 70(4): 562-571.
21. Patrignani, F., Siroli, L., Serrazanetti, DI., Braschi, G., Betoret, E., Reinheimer, JA., et al. (2017) Microencapsulation of functional strains by high pressure homogenization for a potential use in fermented milk. Food Res Int, 97: 250–257.
22. Afzaal, M., Saeed, F., Arshad, MU., Nadeem, MT., Saeed, M., Tufail, T. (2019) The Effect of Encapsulation on The Stability of Probiotic Bacteria in Ice Cream and Simulated Gastrointestinal Conditions. Probiotics Antimicrob Proteins, 11(4): 1348-1354.
23. Kataria, A., Achi, SC., Halami, PM. (2018) Effect of Encapsulation on Viability of Bifidobacterium longum CFR815j and Physiochemical Properties of Ice Cream. Indian J Microbiol, 58(2): 248-251.
24. Terpou, A., Mantzourani, I., Galanis, A., Kanellaki, M., Bezirtzoglou, E., Bekatorou, A., et al. (2018) Employment

of L. paracasei K5 as a Novel Potentially Probiotic Freeze-Dried Starter for Feta-Type Cheese Production. Microorganisms, 7(1): 3.

25. Astesana, DM., Zimmermann, JA., Frizzo, LS., Zbrun, MV., Blajman, JE., Berisvil, AP., et al. (2018) Development and storage studies of high density macrocapsules containing Lactobacillus spp. strains as nutritional supplement in young calves. Rev Argent Microbiol, 50(4): 398-407.

26. FAO-WHO. Food and Agriculture Organization of the United Nations. World Health Organization. 2001. Report of a joint FAO-WHO expert consultation on evaluation of the health and nutritional properties of probiotics in food including powder milk with live lactic acid bacteria, 34 p.

27. Pieniz, S., Andreazza, R., Anghinoni, T., Camargo, F., Brandelli, A. (2014) Probiotic potential, antimicrobial and antioxidant activities of Enterococcus durans strain LAB18s. Food Control, 37: 251-256.

28. Maia, LF, Giazzi, A, Brandalize, C, Katsuda, MS, Rocha, KR, Terra, MR. Furlaneto, MC. (2017). Isolation and characterization of potential probiotic enterococci strains from soft cheese flora. African Journal of Microbiology Research. 11(12):482-87.

29. Cebrián, R., Baños, A., Valdivia, E., Pérez-Pulido, R., Martínez-Bueno, M., Maqueda, M. (2012) Characterization of functional, safety, and probiotic properties of Enterococcus faecalis UGRA10, a new AS-48-producer strain. Food Microbiol, 30(1): 59-67.

30. Bagci, U., Togay, SO., Temiz, A., Ay, M. (2019) Probiotic characteristics of bacteriocin-producing Enterococcus faecium strains isolated from human milk and colostrum. Folia Microbiol (Praha), 64(6): 735-750.

31. Nascimento, LCS., Casarotti, SN., Todorov, SD., Penna, ALB. (2019) Probiotic potential and safety of enterococci strains. Annals of Microbiology, 69: 241–252.

32. Guerra, AF, Lemos Junior, WJF, Santos, GO, Andrighetto, C, Gianomini, A, Corich, V, Luchese, RH. (2018). Lactobacillus paracasei probiotic properties and survivability under stress-induced by processing and storage of ice cream bar or ice-lolly. Ciência Rural. 48(09):1-9.

33. Senter, L, Pereira, JQ, Vieira, MA, Michielin, EMZ, Tondo, EC. (2015). Isolation and characterization of Lactobacillus plantarum BLS29 as a potential probiotic starter culture for pork sausage production. African Journal of Microbiology Research. 9(32):1887-95.

34. Dutra, V., Silva, AC., Cabrita, P., Peres, C., Malcata, X., Brito L. (2016) Lactobacillus plantarum LB95 impairs the virulence potential of Gram-positive and Gram-negative food-borne pathogens in HT-29 and Vero cell cultures. J Med Microbiol, 65(1): 28-35.

35. Zommiti, M., Cambronel, M., Maillot, O., Barreau, M., Sebei, K., Feuilloley, M., et al. (2018) Evaluation of Probiotic Properties and Safety of Enterococcus faecium Isolated From Artisanal Tunisian Meat "Dried Ossban". Front Microbiol, 9: 1685.

36. Barbosa, J., Teixeira, P. (2017) Development of probiotic fruit juice powders by spray drying: a review. Food Reviews International. 33(4): 335-358.

37. Surono, I., Verhoeven, J., Verbruggen, S., Venema, K. (2018) Microencapsulation increases survival of the probiotic Lactobacillus plantarum IS-10506, but not Enterococcus faecium IS-27526 in a dynamic, computer-controlled in vitro model of the upper gastrointestinal tract. J Appl Microbiol, 124(6): 1604-1609.

38. Agência Nacional de Vigilância Sanitária – Gerência Geral de Alimentos. 2017. Probióticos: Construção da Lista de Linhagens Probióticas. Disponível em: <http://portal.anvisa.gov.br/documents/3845226/0/A%C3%A1lise+das+Linhagens+de+Probi%C3%B3ticos__23042018.pdf/6e37da13-2151-4330-85b0-0f449dbb0e95>. Acesso em: 28 out. 2018.