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# Buffalo whey as a source of lactic acid bacteria of biotechnological interest

## *Soro de leite de búfala como fonte de bactéria láctica de interesse biotecnológico*

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**ABSTRACT:** Whey has garnered research interest due to its high nutritional value and environmental impact, as it is a source of microorganisms that can be isolated and characterized for their functional properties. This study aimed to evaluate buffalo whey as a source of lactic acid bacteria (LAB) with biotechnological potential. Buffalo whey was obtained through enzymatic coagulation, and its physicochemical and microbiological properties were analyzed. LAB enumeration and isolation were conducted using selective media, specifically Man, Rogosa, and Sharpe (MRS) and M17. The isolates were identified via MALDI-TOF, and their enzymatic activity, safety, and antimicrobial effects against *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7644, and *Staphylococcus aureus* ATCC 25923 were assessed. The buffalo whey complied with the minimum physicochemical and microbiological standards set by IN No. 94/2020, indicating that good agricultural practices and good manufacturing practices were followed during both milking and processing. A total of 62 LAB strains were isolated and identified via MALDI-TOF-MS, including *Lacticaseibacillus paracasei* (1.61%), *Enterococcus faecium* (4.84%), and *Lactococcus lactis* (93.55%). Of these isolates, 98.38% were proteolytic, 83.87% exhibited lipolytic activity, and none produced lecithinase. The LAB isolates demonstrated antimicrobial activity against the Gram-positive pathogens tested, and 46.77% of the strains were considered innocuous. In summary, buffalo whey presents viable properties and has enabled the isolation of LAB strains with promising potential for applications in bioprocesses and functional studies.

**KEYWORDS:** microbiological analysis; lactic acid bacteria; enzymes; whey.

**RESUMO:** O soro de leite é alvo de estudos devido ao seu alto valor nutricional e potencial poluente, sendo fonte de microrganismos, que podem ser isolados e caracterizados quanto as suas propriedades funcionais. Objetivou-se avaliar o soro de leite de búfala como fonte de bactérias lácticas (BAL) com potencial biotecnológico. O soro de leite de búfala foi obtido através de coagulação enzimática, e teve suas propriedades físico-químicas e microbiológicas caracterizadas. Para contagem e isolamento das BAL foram realizados cultivos nos meios seletivos, Man Rogosa Sharpe (MRS) e M17. Os isolados foram identificados por MALDI-TOF e avaliou-se a atividade enzimática, inocuidade e atividade antimicrobiana frente a *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7644 e *Staphylococcus aureus* ATCC 25923. O soro de leite de búfala atendeu às exigências mínimas dos parâmetros físico-químicos e microbiológicas da IN N° 94/2020, o que demonstra que as boas práticas agropecuárias e as boas práticas de fabricação foram realizadas durante a ordenha e o processamento do soro de leite de búfala, respectivamente. Foram isoladas 62 BAL, identificadas por MALDI-TOF-MS, sendo 1 *Lacticaseibacillus paracasei* (1,61%), 3 *Enterococcus faecium* (4,84%) e 58 *Lactococcus lactis* (93,55%). Das 62 BAL avaliadas, 98,38% demonstraram-se proteolíticas, 83,87% lipolíticas e nenhuma produtora de lecitinase. As BAL (69,35%) demonstraram atividade antimicrobiana contra as bactérias patogênicas Gram-positivas testadas e, 46,77% das BAL foram consideradas inócuas. Portanto, foi possível identificar as propriedades do soro de leite de búfala e isolar BAL com potencial de estudos em bioprocessos e prospecção do potencial funcional.

**PALAVRAS-CHAVE:** análise microbiológica; bactérias ácido lácticas; enzimas; soro de leite.

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## INTRODUCTION

Buffalo milk has gained global significance, particularly due to its unique physicochemical composition, which differs from that of bovine milk. In Brazil, buffalo milk is primarily utilized in the production of derivatives, especially cheese, resulting in the generation of substantial volumes of whey (Godinho *et al.*, 2020). Whey, the largest by-product of the dairy industry, accounts for approximately 85-90% of the milk volume used in cheese production. Given its considerable environmental impact as an effluent, various methods and technologies have been developed for whey management. Despite recent advances in whey utilization, its potential as a source of bacteria with biotechnological applications remains largely unexplored (Rama *et al.*, 2019).

The bacterial concentration in whey typically exceeds that found in milk, a consequence of cheese manufacturing processes that promote bacterial growth, including the use of pasteurized milk and the addition of bacterial starter cultures (Duarte *et al.*, 2020). The microbiota of whey consists primarily of lactic acid bacteria (LAB), which include genera such as *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Enterococcus*, and *Streptococcus* (Rama *et al.*, 2019; Kieliszek *et al.*, 2021).

LAB have been employed for centuries in the production of fermented foods and beverages due to their capacity to impart desirable sensory characteristics, improve digestibility, shorten maturation periods, and inhibit pathogenic and spoilage microorganisms (Tulini *et al.*, 2016). These contributions to food development are attributed to their ability to produce various metabolites, including organic acids, diacetyl, acetoin, volatile compounds, bacteriocins, exopolysaccharides, and proteolytic and lipolytic enzymes (García-Cano *et al.*, 2019; González-González *et al.*, 2022). Beyond their influence on food's physicochemical properties, certain LAB strains are regarded as probiotics due to their beneficial effects on human health (García-Cano *et al.*, 2019).

The isolation and characterization of new LAB strains can advance the food industry through potential applications of these isolates or their metabolites (Rzepkowska *et al.*, 2017). While literature reports on the biotechnological potential of microorganisms isolated from bovine milk and whey are available, knowledge about the microbial composition and biotechnological potential of microorganisms from buffalo whey remains limited. Therefore, given the significance of whey, this study aimed to evaluate buffalo whey as a source of LAB with biotechnological potential.

## MATERIAL AND METHODS

From July to October 2021, five collections of raw buffalo milk were conducted at the Agricultural Experimental Station of the Federal University of Rio Grande do Sul (UFRGS), located in Eldorado do Sul, RS, Brazil. Buffalo milk was obtained following Good Agricultural Practices (GAP) through mechanical milking (Domenico; Motta, 2022) of five multiparous buffalo females (*Bubalus bubalis*) averaging 60 days of lactation, from the Murrah and Mediterranean breeds. Samples were placed in sterile containers within insulated boxes and transported to the Microbiology Laboratory of the Institute of Basic Health Sciences. To obtain buffalo whey, one liter of raw buffalo milk was heated to 40 °C, followed by the addition of HA-LA liquid coagulant (5 mL diluted in 25 mL of water) and calcium chloride (0.5 g). After 30 minutes of resting, the curd was cut and strained, yielding approximately 800 mL of whey and 198.33 g of curd mass.

The buffalo whey was freeze-dried at the Institute of Food Science and Technology at UFRGS and sent to Sooro Renner Nutrition S/A, which carried out the physical-chemical characterization. Fat, acidity, protein, lactose, pH, moisture, ash, chlorides, sodium, and potassium were determined by the Manual of Official Methods for Analysis of Foods of Animal Origin of the Ministry of Agriculture, Livestock, and Supply (Brasil, 2019).

For microbiological characterization, Normative Instruction No. 30/2018 was followed (Brasil, 2018). One milliliter of the whey sample was diluted in 9 mL of 0.85% saline solution, and six serial dilutions were performed in triplicate. Mesophilic microorganism counts were obtained by plating each dilution on Plate Count Agar (PCA; Kasvi, São José dos Pinhais, PR, Brazil) and incubating at 37 °C for 48 h. Results were expressed as the average count, multiplied by the dilution factor, in CFU/mL. Total and thermotolerant coliforms were counted on violet red bile agar (VRBA; Biolog, Belo Horizonte, MG, Brazil) by plating serial dilutions and incubating at 37 °C for 24 h. Confirmatory tests for total coliforms involved inoculating suspected colonies in brilliant green bile broth with 2% lactose (LaborClin, Pinhais, PR, Brazil) and incubating at 37 °C for 24–48 h. For thermotolerant coliforms, suspected colonies were inoculated in EC broth (Kasvi, São José dos Pinhais, PR, Brazil) and incubated at 45 °C in a water bath for 24–48 h. Coagulase-positive *Staphylococcus* counts were obtained by plating serial dilutions on Baird Parker agar (BP; Oxoid, Basingstoke, England) and incubating at 37 °C for 30–48 h, with typical colonies selected for coagulase testing using rabbit plasma (LaborClin), incubated at 37 °C for 6 h. *Listeria monocytogenes* detection was conducted using two-stage selective enrichment. First, UVM broth (BD Difco, Le Pont de Claix, France) and then Fraser broth (Merck, Darmstadt, Germany) were incubated at 30 °C for 24 h each. Isolation was conducted on Oxford agar (OA; Himedia, Mumbai, India) at 30 °C for 24 h, where black colonies surrounded by a black halo indicated the presence of *L. monocytogenes*. *Salmonella spp.* detection involved pre-enrichment in 1% buffered peptone saline solution at 37 °C for 16–20 h, followed by selective enrichment in Rappaport Vassiliadis broth (Merck, Darmstadt, Germany) and Selenite-Cystine broth (Himedia, Mumbai, India), incubated in a water bath at 40 °C for 24 h. Isolation was conducted on XLD agar (Kasvi,

São José dos Pinhais, PR, Brazil) at 37 °C for 24 h, with pink colonies indicating the presence of *Salmonella*.

LAB enumeration and isolation were performed by plating serial dilutions on Man, Rogosa, and Sharpe agar (MRS; Merck, Darmstadt, Germany) and M17 agar (Oxoid, Basingstoke, England), incubated aerobically at 37 °C for 48 h and anaerobically at 30 °C for 48 h. LAB counts were reported in CFU/mL. Colonies with morphological characteristics of LAB were selected and preliminarily characterized through Gram staining and catalase testing. Only Gram-positive and catalase-negative cultures, characteristic of LAB, were selected for further identification.

Selected cultures were identified using Matrix-Assisted Laser Desorption Ionization – Time of Flight (MALDI-TOF) mass spectrometry via direct transfer. This method involved placing a small culture sample on a MALDI-TOF target plate spot, which was then covered with α-cyano-4-hydroxycinnamic acid (HCCA) matrix. Analyses were conducted using MALDI Biotyper 4.0 (Bruker Daltonics, Bremen, Germany), and after obtaining the sample spectrum, MBT RTC (Real Time Classification) software identified the best match by comparing the sample with stored spectra, generating results with varying reliability scores. Scores ≥2,000 indicated species-level identification, scores of 1,700–1,999 indicated genus-level identification, and scores <1,700 were interpreted as no identification.

Enzymatic properties were assessed following Bogo *et al.* (2017), with modifications. Proteolytic activity was tested by inoculating bacterial cultures onto milk agar (tryptone soy agar – TSA (Kasvi, São José dos Pinhais, PR, Brazil) + 10% bovine skim milk) and incubating at 30 °C for 48 h, with *Staphylococcus aureus* ATCC 25923 as a positive control. Lipolytic activity was tested on tributyrin agar [peptone (5 g/L), agar (15 g/L), yeast extract (3 g/L), and tributyrin (10 mL/L)], incubated at 30 °C for 48 h, with *Candida parapsilosis* ATCC 22019 as a positive control. Lecithinase production was tested by inoculating bacteria on BP agar supplemented with 10% egg yolk emulsion (NewProv, Pinhais, PR, Brazil) and incubating at 30 °C for 48 h, with *S. aureus* ATCC 25923 as a positive control. Opaque zones around colonies indicated enzyme production, and results were measured in millimeters (mm). Enzymatic activities were classified by halo size: very high (>10 mm), high (3–10 mm), low (<3 mm), and absent (no halo formation) (Silva *et al.*, 2020). All tests were performed in triplicate.

LAB antimicrobial activity against *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7644, and *S. aureus* ATCC 25923 was evaluated via the sting method (Coman *et al.*, 2014). Pathogenic cultures were prepared in 0.85% saline according to the McFarland Scale of 0.5 ($10^8$ CFU/mL). Each suspension was swabbed on Muller Hinton agar plates (MH; BD, Franklin Lakes, USA), and LAB were then inoculated using a platinum needle. Pathogen susceptibility to LAB was assessed by measuring the inhibition zone diameter (mm) around LAB after incubation at 37 °C for 24 h. Experiments were conducted in triplicate.

For hemolytic activity, isolates were inoculated on Columbia Blood Agar (CBA; Himedia, Mumbai, India) supplemented with 5% defibrinated sheep blood (New Prov) and incubated at 37 °C for up to 48 h. Plates were examined for β-hemolysis (clear zones around colonies), α-hemolysis (greenish zones around colonies), or γ-hemolysis (no zones) (Breyer *et al.*, 2021). Gelatin hydrolysis was tested by inoculating isolates with a platinum needle into nutrient gelatin tubes, incubated at 37 °C for 24 h, then held at 4 °C for 20 min to assess gelatinase activity by examining medium consistency. *S. aureus* ATCC 25923 served as a positive control for both tests (Dias; Fusieger; Motta, 2019).

Statistical analysis for mesophilic microorganisms and LAB counts in buffalo whey was performed using R software via a one-way analysis of variance (ANOVA) with a significance level of $p < 0.05$. Multiple comparisons were conducted using Tukey's test ($p < 0.05$).

## RESULTS AND DISCUSSIONS

The buffalo whey exhibited physicochemical values (Table 1) that align with the standards set by Normative Instruction No. 94/2020 (Brasil, 2020). The whey showed a neutral pH (6.49), producing a sweet whey as expected, given its derivation through enzymatic coagulation. Sweet whey is generated by milk coagulation using rennet, an enzyme mixture containing the protease chymosin, in contrast to acid whey, which is produced when milk coagulates through the action of lactobacilli or the addition of mineral acids (e.g., hydrochloric or sulfuric acid) or organic acids (e.g., lactic acid) (Rama *et al.*, 2019).

The fat content found in the present study (1.47%) was lower than the values reported by Rathour *et al.* (2016) and Argenta *et al.* (2021) for concentrated buffalo whey (6.50%)

**Table 1.** Physicochemical analysis of freeze-dried buffalo milk whey.*

| Parameters | Composition |
|---|---|
| Moisture (%) | 2,92 ± 0,79 |
| Fat (%) | 1,47 ± 0,79 |
| Acidity (g lactic acid) | 0,985 ± 0,07 |
| pH | 6,49 ± 0,05 |
| Ash (%) | 5,65 ± 0,30 |
| Chlorides (%) | 1,17 ± 0,14 |
| Sodium (mg/100g) | 612 ± 4,61 |
| Potassium (mg/100g) | 1238 ± 10,06 |
| Protein (%) | 12,77 ± 0,51 |
| Lactose (%) | 76,95 ± 1,43 |

*Data were expressed as the mean and standard deviation of the triplicates performed.

and buffalo whey powder (4.97%), respectively. The protein content obtained for buffalo whey (12.77%) was close to those reported by Merkel, Voropaeva, and Ondrusek (2021) for bovine whey (9.2%) and similar to that reported by Argenta *et al.* (2021) for buffalo whey (10.89%). Whey composition depends on factors such as the milk's origin, the feeding practices for producing animals, lactation stage, post-milking storage conditions, heat treatment, and casein precipitation method (Duarte *et al.*, 2020; García-Cano *et al.*, 2019). Additionally, in the present study, the lactose content (76.95%) was similar to that reported by Merkel, Voropaeva, and Ondrusek (2021) for bovine whey (73%) and higher than that reported by Argenta *et al.* (2021) for buffalo whey (46.26%). Therefore, processing liquid whey to develop new products, such as dairy beverages, or to create value-added products like whey powder, concentrated whey (WC), whey protein concentrate (WPC), and whey protein isolates (WPI) is advantageous for the dairy industry (Kieliszek *et al.*, 2021; Rathour *et al.*, 2016).

The microbiological characterization results for buffalo whey are presented in Table 2. The mesophilic microorganism counts ranged from 2.50 x $10^5$ to 2.37 x $10^8$ CFU/mL, exceeding the limits established by IN No. 94/2020 (Brasil, 2020). This elevated mesophilic count can be attributed to the absence of pasteurization in the buffalo milk used for whey production. In a study by Desconsi, Izário Filho, and Salazar (2014), mesophilic microorganism counts were reported as >3.0 x $10^3$ CFU/mL for silo-stored whey and 5.40 x $10^2$ CFU/mL for pasteurized whey, indicating that pasteurization effectively reduces microbial load in whey. High mesophilic counts typically signal suboptimal conditions during milk production, storage, and processing, as well as insufficient equipment sanitation or improper temperature/time parameters during whey production and storage (Duarte *et al.*, 2020; Mendes; Souza, 2017).

The presence of coliforms in food indicates contamination during the manufacturing process or even post-processing. However, in the present study, total and thermotolerant coliform counts were below the limits recommended by IN No. 94/2020 (Brasil, 2020). Within the method's limitations, total and thermotolerant coliform counts were recorded as <10 CFU/mL and <3 CFU/mL, respectively. Similar results were reported by Desconsi, Izário Filho, and Salazar (2014), who found no coliform levels in buffalo milk whey that exceeded legislative standards. Furthermore, the coliform counts in this study were more favorable than those in studies by Teixeira, Fonseca and Menezes (2007) and Mendes and Souza (2017), indicating that Good Manufacturing Practices (GMP) were followed during buffalo whey processing.

In the analysis of coagulase-positive *Staphylococcus*, growth of typical colonies was observed only in the last two samples (9x$10^4$ CFU/mL and 1.63x$10^5$ CFU/mL). However, confirmatory tests indicated negative results for coagulase-positive *Staphylococcus*, meeting the standards of IN No. 94/2020 (Brasil, 2020), with the result expressed as <10 CFU/mL due to technical limitations. However, growth of atypical colonies was observed in all samples, varying between 6.67x$10^5$ and 2.27x$10^7$ CFU/mL. Mendes and Souza (2017) obtained similar results for bovine milk whey, with no samples exhibiting coagulase-positive *Staphylococcus*. Conversely, Teixeira, Fonseca and Menezes (2007) identified coagulase-positive *Staphylococcus* in whey samples. The presence of this microorganism indicates inadequate product handling, as well as poor hygiene conditions of the handler (Mendes; Souza, 2017). In this research, the presence of *Listeria monocytogenes* and *Salmonella* spp. were not detected in buffalo milk whey, meeting the standards required by IN No. 94/2020 (Brasil, 2020). This finding highlights the absence of pathogens and supports the quality of buffalo whey, a factor of considerable interest to the dairy industry, given that the presence of these pathogens can prompt major public health concerns. *L. monocytogenes* and *Salmonella*, often found on food-handling surfaces, have been linked to several foodborne outbreaks, including listeriosis and salmonellosis, primarily transmitted through contaminated foods such as soft cheeses, ice cream, unpasteurized milk, meats, vegetables, and seafood (Teixeira; Fonseca; Menezes, 2007; Mendes; Souza, 2017; Duarte *et al.*, 2020). Despite the high counts of mesophilic microorganisms in the buffalo whey samples, pathogen presence was ruled out, confirming compliance with microbiological standards. It is suggested that the high mesophilic counts may be associated with the presence of LAB in buffalo whey. A whey sample with sound microbiological quality ensures food safety, reduces the

**Table 2.** Results of the microbiological analysis of buffalo milk whey. *

| Microorganisms | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| Mesophilic aerobes (CFU/mL) | 2,50x$10^{5\,a}$ | 2,5x$10^{7\,a,b}$ | 2,37x$10^{8\,c}$ | 6,37x$10^{7\,a,b}$ | 2,04x$10^{8\,c}$ |
| Total coliforms (CFU/mL) | <10 | <10 | <10 | <10 | <10 |
| Thermotolerant coliforms (CFU/mL) | <3 | <3 | <3 | <3 | <3 |
| Coagulase-positive *Staphylococcus* (CFU/mL) | <10 | <10 | <10 | <10 | <10 |
| *Listeria monocytogenes* | Absence | Absence | Absence | Absence | Absence |
| *Salmonella* sp | Absence | Absence | Absence | Absence | Absence |

* Data were expressed as the average of triplicate count. Equal letters on the same line indicate the absence of statistical difference (p<0.05).

risk of foodborne diseases, and enhances its potential as an ingredient for developing new food products.

The LAB count in buffalo whey was measured on MRS and M17 agars at varying incubation temperatures (30 °C and 37 °C) under both aerobic and anaerobic conditions, with LAB growth observed under all conditions (Table 3). Marasco *et al.* (2022) conducted a similar study with LAB counts under diverse conditions: on MRS agar under aerobic and anaerobic conditions at 30 °C and 44 °C for mesophilic and thermophilic lactobacilli, respectively; on ESTY agar under aerobic and anaerobic conditions at 30 °C and 44 °C for mesophilic and thermophilic cocci, respectively; and on BHI agar under aerobic conditions at 37 °C for enterococci. Marasco *et al.* (2022) reported comparable results, observing LAB growth across all conditions for both buffalo and bovine whey samples.

Moreover, the buffalo whey in this study was produced in the laboratory without starter cultures, as the goal was to characterize the indigenous lactic microbiota. Understanding new LAB strains isolated from dairy products is highly valuable to the food industry, as it can support the development of new starter cultures and functional bacterial strains (Bao *et al.*, 2012). In this study, 83 LAB colonies were isolated based on morphological characteristics, with 62 of these isolates identified as Gram-positive and catalase-negative, as detailed in Table 4.

A total of 62 LAB isolates were analyzed using MALDI-TOF/MS, identifying 58 (93.55%) as *Lactococcus lactis*, 3 (4.84%) as *Enterococcus faecium*, and 1 (1.61%) as *Lacticaseibacillus paracasei* (Figure 1a). All samples were predominantly composed of the *Lactococcus* genus (Figure 1b), similar to the findings on homemade Feta cheese samples reported by Papadimitriou *et al.* (2022). The results of this study align with those of Marasco *et al.* (2022), who also identified a high prevalence of *Lactococcus lactis* subsp. *lactis* in buffalo milk whey (19 isolates) and cow milk whey (18 isolates). Additionally, Riquelme *et al.* (2015) observed a 77% prevalence of *Lactococcus lactis* in Pico cheese. A similar predominance of lactococci over lactobacilli was found in yak dairy products, where *Lactococcus* accounted for 51.41% of the isolates (Bao *et al.*, 2012). In contrast, Duarte *et al.* (2020) observed the genera *Streptococcus* and *Lactobacillus* in whey samples from four dairies in Italy.

The microbial composition of whey can vary widely, as it is directly influenced by the dairy matrix used in cheese production. Notably, LAB form part of the microbiota throughout the milk production process. From the moment milk leaves the udder, it comes into contact with LAB, other microorganisms, and potential pathogens that may be present on the udder surface, in milking equipment, transport and storage containers, and within the dairy production environment. In artisanal production facilities, the microbial composition of whey can further vary due to processing under environmental conditions that are not strictly controlled (Rzepkowska *et al.*, 2017; Marasco *et al.*, 2022). Characterizing the lactic microbiota of whey, therefore, aims to identify LAB species that contribute to maintaining the quality and specific characteristics of the final product. Selected microorganisms can then be evaluated for their metabolic and technological properties, particularly as the market continues to seek microbial strains that can enhance the sensory and organoleptic qualities of dairy products, as well as their shelf life. Additionally, new isolates may be assessed for their probiotic potential, which is of significant interest to the food sector given the rising demand for products that offer nutritional value and provide health benefits to consumers (Marasco *et al.*, 2022).

The enzymatic activity of the 62 LAB isolated from buffalo whey was evaluated, with results presented in Table 4. Proteolytic activity was observed in 98.38% (n=61) of isolates, which exhibited a degradation halo indicative of proteolytic enzyme production. Only *L. lactis* 55A5-2 showed no proteolytic activity when cultured on milk agar. The findings of this study align with research showing that the majority of LAB strains exhibit proteolytic activity (Bogo *et al.*, 2017; Araújo-Rodrigues *et al.*, 2021; Breyer *et al.*, 2021). However, studies by García-Cano *et al.* (2019) and Silva *et al.* (2020) reported a lower incidence of proteolytic LAB in dairy products, at 61.3% and 48.6% of isolates, respectively. The LAB proteolytic system can hydrolyze milk proteins, converting them into peptides and amino acids essential for bacterial growth. This system plays a critical role in the production of various metabolites that contribute to the aroma and flavor of fermented products (Tulini *et al.*, 2016; Dias; Fusieger; Motta, 2019; Kieliszek *et al.*, 2021). In cheeses with internal and superficial ripening, for example, it is essential that proteolysis occurs (Silva *et al.*, 2020). In addition to their influence on the sensory properties of fermented products, some LAB strains are known to release bioactive peptides with antimicrobial, antihypertensive, and antioxidant properties (González-González *et al.*, 2022).

Regarding the lipolytic profile, 83.87% (n=52) of the isolates demonstrated lipolytic potential in tributyrin agar

**Table 3.** LAB counts in buffalo milk whey carried out on MRS and M17 agar under aerobic conditions at 37 °C and anaerobic conditions at 30 °C. *

| Culture conditions | Sample 1 | Sample 2 | Sample 3 | Sample 4 | Sample 5 |
|---|---|---|---|---|---|
| MRS aerobiosis (CFU/mL) | $2{,}85\times10^{5\,a}$ | $1{,}18\times10^{7\,b}$ | $2{,}18\times10^{7}$ | $1{,}04\times10^{7\,b}$ | $2{,}03\times10^{6\,a}$ |
| MRS anaerobiosis (CFU /mL) | $2{,}6\times10^{4\,a}$ | $1{,}04\times10^{7\,a,b}$ | $2{,}31\times10^{7}$ | $1{,}34\times10^{7\,b}$ | $2{,}1\times10^{6\,a,b}$ |
| M17 aerobiosis (CFU /mL) | $2{,}8\times10^{5\,a}$ | $2{,}84\times10^{7\,a,b}$ | $9{,}63\times10^{7\,c}$ | $6{,}53\times10^{7\,b,c}$ | $1{,}74\times10^{8}$ |
| M17 anaerobiosis (CFU/mL) | $2{,}7\times10^{4}$ | $2{,}7\times10^{7}$ | $1{,}13\times10^{8\,a}$ | $7{,}9\times10^{7}$ | $1{,}21\times10^{8\,a}$ |

* Data were expressed as the average of triplicate counts. Equal letters on the same line indicate the absence of statistical difference ($p<0.05$).

**Table 4.** Enzymatic activity and antimicrobial activity of LAB isolated from buffalo whey. [a]

| Microorganism | Enzymatic activity [b] | | Antimicrobial activity [c] | |
|---|---|---|---|---|
| | PA | LA | *L. monocytogenes* | *S. aureus* |
| *E. faecium* 12B0-1 | 9,67 ± 0,58 | 8,33 ± 0,55 | - | - |
| *L. paracasei* 12B0-2 | 17,00 ± 1,00 | - | - | - |
| *L. lactis* 22B1 | 10,67 ± 0,58 | 4,00 ± 1,00 | 10,33 ± 0,55 | 4,33 ± 0,58 |
| *E. faecium* 22C2-1 | 11,67 ± 2,89 | 4,67 ± 0,50 | 9,00 ± 1,00 | 4,00 ± 0,10 |
| *L. lactis* 22C2-2 | 12,00 ±2,65 | - | 9,33 ± 0,58 | 4,33 ± 0,50 |
| *L. lactis* 23A0-1 | 10,00 ± 1,00 | 5,00 ± 1,00 | 9,00 ± 0,10 | 3,00 ± 0,10 |
| *L. lactis* 23A0-2 | 5,33 ± 0,58 | 3,33 ± 0,55 | 10,67 ± 1,15 | 4,00 ± 0,10 |
| *L. lactis* 23A1-1 | 11,00 ± 1,00 | 4,00 ± 0,00 | 9,33 ± 0,60 | 4,67 ± 0,58 |
| *L. lactis* 23A1-2 | 3,33 ± 0,58 | 5,00 ± 0,00 | 10,33 ± 1,53 | 4,33 ± 0,60 |
| *L. lactis* 25A1-2 | 19,67 ± 1,53 | 4,00 ± 0,00 | - | 2,67 ± 0,60 |
| *L. lactis* 32A4-1 | 15,33 ± 0,58 | - | 10,00 ± 0,10 | 4,33 ± 0,55 |
| *L. lactis* 32A4-2 | 3,00 ± 0,00 | 3,67 ± 0,58 | 7,33 ± 0,58 | 5,00 ± 0,10 |
| *L. lactis* 32A4-3 | 9,00 ± 1,00 | 3,33 ± 0,55 | - | - |
| *L. lactis* 32B4-1 | 22,67 ± 0,50 | 4,33 ± 0,60 | - | - |
| *L. lactis* 32B4-2 | 8,00 ± 1,00 | 4,00 ± 0,00 | - | - |
| *L. lactis* 32B4-3 | 11,33 ± 0,58 | 5,00 ± 0,10 | 14,00 ± 1,00 | 4,33 ± 0,58 |
| *L. lactis* 32C4-1 | 4,00 ± 1,00 | 5,00 ± 0,01 | - | - |
| *L. lactis* 32C4-2 | 2,33 ± 0,58 | 5,00 ± 0,00 | - | - |
| *L. lactis* 32C4-3 | 8,33 ± 1,53 | 4,33 ± 0,55 | 9,00 ± 0,10 | 4,67 ± 0,55 |
| *L. lactis* 32C4-4 | 15,67 ± 1,23 | 3,00 ±0,10 | 8,33 ± 0,50 | 4,33 ± 0,55 |
| *L. lactis* 33A4-1 | 5,33 ± 0,55 | 4,33 ± 0,55 | - | - |
| *L. lactis* 33A4-2 | 3,67 ± 0,55 | 4,00 ± 0,00 | - | - |
| *L. lactis* 33A4-3 | 15,33 ± 0,58 | 3,00 ± 0,10 | 11,33 ± 0,60 | 5,00 ± 0,10 |
| *L. lactis* 33A4-4 | 3,00 ± 0,00 | 3,67 ± 0,60 | 6,67 ± 0,58 | 4,00 ± 0,10 |
| *L. lactis* 35A4-1 | 9,67 ± 2,80 | 5,33 ± 0,58 | - | - |
| *L. lactis* 42A5-1 | 10,67 ±0,58 | 3,67 ± 0,55 | 10,00 ± 1,00 | 3,67 ± 0,58 |
| *L. lactis* 42A5-2 | 8,67 ± 0,56 | 4,33 ± 0,58 | 6,33 ± 1,15 | 3,33 ± 0,58 |
| *L. lactis* 42B5-1 | 8,00 ± 0,00 | 8,33 ± 1,53 | - | - |
| *L. lactis* 42B5-2 | 13,33 ± 0,58 | 4,33 ± 0,50 | - | - |
| *L. lactis* 42B5-3 | 13,00 ± 1,00 | - | 8,00 ± 0,10 | 4,33 ± 0,50 |
| *L. lactis* 42C5-1 | 11,00 ± 1,00 | 4,33 ± 0,58 | 9,33 ± 0,58 | 4,33 ± 0,50 |
| *E. faecium* 43A4-1 | 12,67 ± 0,50 | 5,00 ± 0,10 | 9,67 ± 0,55 | - |
| *L. lactis* 43A4-2 | 4,33 ± 0,55 | 3,33 ± 0,50 | 8,33 ± 0,58 | 4,00 ± 0,10 |
| *L. lactis* 43A5-1 | 17,00 ± 1,00 | 4,00 ± 0,00 | 10,33 ± 0,60 | 4,00 ± 0,10 |
| *L. lactis* 43A5-2 | 5,33 ± 0,58 | 5,67 ± 0,60 | 8,00 ± 1,00 | 3,67 ± 0,58 |
| *L. lactis* 43A5-3 | 13,33 ± 1,15 | 4,67 ± 0,55 | 12,00 ± 1,00 | 4,33 ± 0,60 |
| *L. lactis* 43A5-4 | 12,33 ± 0,60 | 4,00 ± 0,10 | 8,67 ± 0,55 | 4,00 ± 0,10 |
| *L. lactis* 43A5-5 | 10,67 ± 0,50 | 4,00 ± 0,00 | 7,33 ± 0,58 | 4,67 ± 0,55 |
| *L. lactis* 44C5-1 | 9,33 ± 1,53 | 4,67 ± 0,50 | 10,00 ± 1,00 | 3,33 ± 0,58 |
| *L. lactis* 44C5-2 | 3,33 ± 0,58 | - | 9,33 ± 1,53 | 4,00 ± 0,10 |
| *L. lactis* 44C5-3 | 10,00 ± 0,00 | 5,00 ± 0,00 | 9,67 ± 0,60 | 3,33 ± 0,58 |
| *L. lactis* 45A5-1 | 3,67 ± 0,62 | 4,00 ± 1,00 | 12,67 ± 1,50 | 5,33 ± 0,55 |

Continue...

**Table 4.** Continuation.

| Microorganism | Enzymatic activity [b] | | Antimicrobial activity [c] | |
|---|---|---|---|---|
| | PA | LA | *L. monocytogenes* | *S. aureus* |
| *L. lactis* 52A4 | 13,67 ±0,55 | 4,00 ± 1,00 | - | - |
| *L. lactis* 52C4 | 10,33 ± 1,15 | 3,00 ± 0,10 | 9,67 ± 0,58 | 3,00 ± 0,10 |
| *L. lactis* 52C5 | 6,00 ± 0,00 | 4,00 ± 1,00 | 10,67 ± 1,10 | 4,00 ± 0,10 |
| *L. lactis* 52B4-1 | 8,67 ± 0,58 | 4,00 ± 0,20 | - | - |
| *L. lactis* 52B4-2 | 9,67 ± 0,55 | 11,00 ± 0,10 | - | - |
| *L. lactis* 52B5-1 | 7,67 ± 0,58 | 4,67 ± 0,58 | 11,67 ± 0,55 | 5,00 ± 0,10 |
| *L. lactis* 53A4-2 | 15,33 ± 1,15 | 4,33 ± 0,60 | 10,33 ± 1,15 | 4,33 ± 0,58 |
| *L. lactis* 53A5-1 | 15,33 ± 0,50 | - | 14,67 ± 0,58 | 4,67 ± 0,55 |
| *L. lactis* 53A5-3 | 15,67 ± 1,15 | 4,67 ± 0,58 | - | - |
| *L. lactis* 54A5 | 11,33 ± 1,50 | 3,33 ± 0,55 | 7,67 ± 0,60 | 4,33 ± 0,58 |
| *L lactis* 54B5-1 | 9,00 ± 0,00 | 5,00 ± 0,10 | 11,00 ± 1,00 | 4,00 ± 0,10 |
| *L. lactis* 54B5-2 | 3,67 ± 0,60 | - | 13,67 ± 0,58 | 4,00 ± 1,00 |
| *L. lactis* 54C5 | 9,00 ± 1,00 | 3,00 ± 0,10 | 11,33 ± 0,55 | 5,00 ± 1,00 |
| *L. lactis* 54C6 | 9,00 ± 1,00 | - | 11,33 ± 0,50 | 4,67 ± 1,15 |
| *L. lactis* 55A5-1 | 7,67 ± 0,55 | 5,00 ± 0,10 | 8,67 ± 0,55 | 4,67 ± 0,60 |
| *L. lactis* 55A5-2 | - | 5,67 ± 1,15 | 13,33 ± 1,15 | 4,33 ± 0,55 |
| *L. lactis* 55A5-3 | 7,67 ± 0,58 | - | 11,00 ± 0,10 | 4,33 ± 0,50 |
| *L. lactis* 55A6-1 | 8,33 ± 0,60 | - | 13,00 ± 1,00 | 4,00 ± 0,10 |
| *L. lactis* 65A5-1 | 11,67 ± 0,58 | 17,00 ± 1,00 | - | - |
| *L. lactis* 65A5-2 | 6,33 ± 0,50 | 8,67 ± 0,10 | - | - |

[a] Data were expressed as the mean and standard deviation of the triplicates performed.
[b] PA=Proteolytic activity; LA=Lipolytic activity; Lecithinase activity was not detected.
[c] Antimicrobial activity was tested against *Escherichia coli* ATCC 10536, *Pseudomonas aeruginosa* ATCC 27853, *Listeria monocytogenes* ATCC 7644 and *Staphylococcus aureus* ATCC 25923. LAB strains tested showed no inhibitory halo against *E. coli* and *P. aeruginosa*.

(Table 4). García-Cano *et al.* (2019) found that 50.3% of the LAB isolated from dairy products exhibited lipolytic activity, with emphasis on 5 *Lactobacillus* spp. and 2 *Pediococcus* spp., which showed the highest activities. The evaluation of lipolytic activity of 11 LAB isolated from Serpa cheese revealed that only 4 LAB from the genus *Lactobacillus* spp. demonstrated enzymatic activity (Araújo-Rodrigues *et al.*, 2021). In contrast, the study by Colombo, Nero and Todorov (2020), found no lipolytic activity among LAB isolates from dairy products. Lipases are enzymes responsible for hydrolyzing triglycerides into free fatty acids, glycerol, and mono- and diglycerides, which contribute to flavor development. These intermediates emulsify other food components, aiding in the texture formation of the final product (Motta; Gomes, 2015; Tanasupawat; Phoottosavako; Keeratipibul, 2015). Lipolytic activity is particularly advantageous for LAB starter cultures, as it breaks down milk fat during maturation to prompt aroma production without giving the product a rancid taste (García-Cano *et al.*, 2019; Motta; Gomes, 2015). In this sense, the degree of lipolysis is an important factor in determining the selection of strains as starter cultures. Furthermore, this property becomes pertinent for LAB vegetable fermentation,

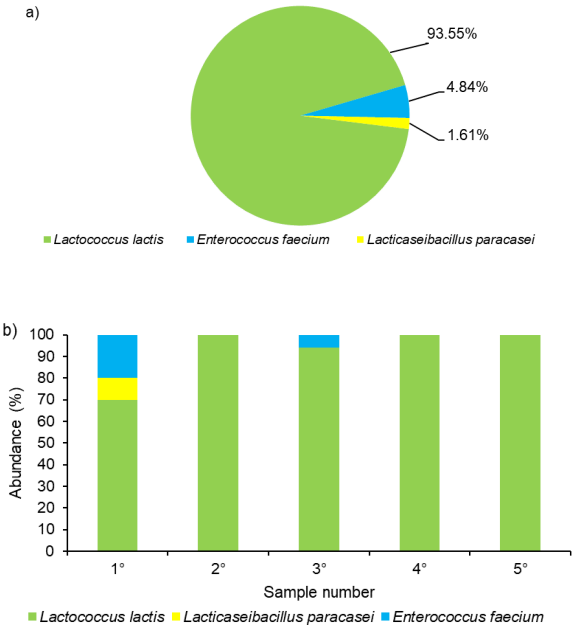


**Figure 1.** a) MALDI-TOF identification of microorganisms isolated and selected from buffalo whey, and b) Taxonomic profile of buffalo whey samples at the genus level.

applications in baked foods, flavor development in fermented dairy products (García-Cano *et al.*, 2019) and fermented meat products (Tanasupawat; Phoottosavako; Keeratipibul, 2015).

The 62 LAB isolates from buffalo whey were tested for their antimicrobial activity against four bacteria associated with foodborne illnesses (Table 4). In total, 69.35% (n=43) of the isolates displayed antimicrobial activity against *L. monocytogenes* ATCC 7644 (with inhibition zones ranging from 6.33 to 13.67 mm) and *S. aureus* ATCC 25923 (with zones ranging from 2.67 to 5.33 mm), both Gram-positive bacteria. However, no antimicrobial activity was observed against the Gram-negative *E. coli* ATCC 10536 or *P. aeruginosa* ATCC 27853. The absence of inhibitory activity of LAB against Gram-negative bacteria has also been reported by Tulini *et al.* (2016), likely due to the protective role of the outer membrane in Gram-negative cells. This study's results surpass those of Silva *et al.* (2020) and Atanasova, Moncheva and Ivanova (2014), who found antimicrobial activity against *L. monocytogenes* ATCC 7644 in only 2.70% (n=1) and 3.22% (n=2) of their isolates, respectively. However, in another study, 90% (n=56) of LAB isolates from fermented fish demonstrated inhibition against *L. monocytogenes* (Desniar *et al.*, 2013). These findings hold relevance for the food industry, as isolates with antimicrobial activity against *L. monocytogenes* could be evaluated for their viability in food applications to prevent listeriosis (Silva *et al.*, 2020). The activity against *S. aureus* ATCC 25923 observed here is consistent with Desniar *et al.* (2013), who reported that 66% (n=41) of isolates exhibited antimicrobial activity against *S. aureus*. Studies by Atanasova, Moncheva, and Ivanova (2014) and Cui *et al.* (2018) obtained inferior results, with only four out of 62 LAB isolates and 37 out of 86 LAB isolates, respectively, displaying antimicrobial activity against *S. aureus*. Preliminary assays suggest the presence of LAB metabolites with antimicrobial effects, such as organic acids, hydrogen peroxide, or bacteriocins (Tulini *et al.*, 2016; Cui *et al.*, 2018). Further research is needed to identify the specific metabolite responsible for this activity.

LAB's ability to hydrolyze gelatin was also evaluated, with only 6.45% (n=4) of isolates showing gelatinase activity, all from the genus *Lactococcus* spp. This finding contrasts with those of Colombo, Nero, and Todorov (2020) and Breyer *et al.* (2021), where no isolate showed gelatinase activity. Regarding hemolytic activity, 50% (n=31) of the isolates analyzed were γ-hemolytic, 46.77% (n=29) were β-hemolytic and 3.23% (n=2) were α-hemolytic. The LAB analyzed by other authors did not show β-hemolysis or α-hemolysis (Cui *et al.*, 2018; Colombo; Nero; Todorov, 2020; Breyer *et al.*, 2021). Therefore, from the 62 LAB analyzed, it could be concluded that 46.77% (n=29) are considered innocuous (1 *Lacticaseibacillus paracasei*, 3 *Enterococcus faecium* and 25 *Lactococcus lactis*), meeting the safety prerequisites for selection as potential probiotic LAB for subsequent application in the food industry (Cui *et al.*, 2018; Breyer *et al.*, 2021).

## CONCLUSIONS

Good agricultural practices and good manufacturing practices were followed during the milking of buffalo milk and the processing of buffalo whey, as the buffalo whey produced in this study met the physicochemical and microbiological standards outlined in IN No. 94/2020, with the exception of mesophilic microorganism counts, which exceeded the permitted level. This increase is likely attributable to the high LAB counts in buffalo milk whey, from which 62 LAB isolates were obtained and identified by MALDI-TOF-MS as *Lactococcus lactis* (93.55%), *Enterococcus faecium* (4.84%), and *Lacticaseibacillus paracasei* (1.61%). Of these 62 LAB isolates, 98.38% exhibited proteolytic activity, 83.87% displayed lipolytic activity, and none produced lecithinase. Additionally, 69.35% of the LAB demonstrated antimicrobial activity against the Gram-positive pathogenic bacteria tested (*L. monocytogenes* ATCC 7644 and *S. aureus* ATCC 25923), while 46.77% of the LAB were classified as innocuous. These findings underscore the importance of isolating, selecting, and characterizing new LAB strains, which are essential for the development of new starter cultures and for identifying LAB with potential applications in bioprocesses and functional product research.

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