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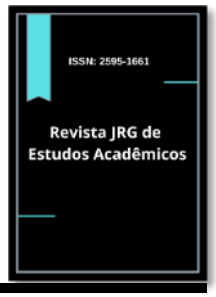

Probiotic potential and technological properties of lactic acid bacteria isolated from Minas Frescal cheese

Potencial probiótico e propriedades tecnológicas de bactérias lácticas isoladas de queijo Minas Frescal

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Abstract
Lactic acid bacteria (LAB) are recognized for their biotechnological and industrial potential, particularly due to the production of bacteriocins, organic acids, and hydrogen peroxide, compounds associated with antimicrobial activity and applicable as natural preservatives. The isolation of LAB from foods, especially milk and dairy products, is strategic for the development of new probiotic products. This study investigated the occurrence and probiotic potential of LAB isolated from Minas Frescal cheese. Biochemical identification included Gram staining, catalase test, gas production from glucose, arginine hydrolysis, and growth under different temperatures and NaCl concentrations. Probiotic and technological assessments included inhibition assays against *Staphylococcus aureus* and *Escherichia coli*, tolerance to acidic pH values (7.0, 3.0, and 2.5), and bile salt concentrations (0.3 - 0.7%), as well as analyses of enzymatic and metabolic activity. All isolates were Gram-positive, catalase-negative, and mostly belonged to the genus *Leuconostoc* (73.3%). Regarding antimicrobial susceptibility, 96.6% of the isolates were sensitive to ampicillin, and 3.3% were sensitive to imipenem. Strong inhibitory activity was observed against *S. aureus* and *E. coli;* however, the cell-free supernatant did not exhibit antagonistic effects. Most strains (96.6%) survived at pH 2.5, and all tolerated bile salts. High proteolytic activity (>97%) was detected, whereas production of diacetyl, capsule, lipase, and hydrogen peroxide was absent. Lactic fermentation occurred in 96.6% of the strains, characterized by the formation of a gel. In conclusion, LAB isolated from Minas Frescal cheese exhibited promising probiotic and technological properties, highlighting their potential application in functional foods with benefits for gut health and food safety."

Keywords: Probiotic potential. Functional foods. Antimicrobial activity. *Leuconostoc*. Dairy products.

Resumo
Bactérias ácido-láticas (BAL) se destacam por seu potencial biotecnológico e industrial devido à produção de bacteriocinas, ácidos orgânicos e peróxido de hidrogênio, compostos associados à atividade antimicrobiana e aplicáveis como conservantes naturais. A busca por BAL isoladas de alimentos, especialmente leite e derivados, é estratégico para o desenvolvimento de novos produtos probióticos. Este estudo investigou a presença e o potencial probiótico de BAL em amostras de queijo Minas Frescal. A identificação bioquímica incluiu coloração de Gram, teste da catalase, produção de gás a partir de glicose, hidrólise de arginina e crescimento em diferentes temperaturas e concentrações de NaCl. As avaliações probióticas e tecnológicas compreenderam ensaios de inibição contra Staphylococcus aureus e Escherichia coli, tolerância a diferentes valores de pH (7,0; 3,0; 2,5) e concentrações de sais biliares (0,3% - 0,7%), bem como análises de atividade enzimática e metabólica. Todos os isolados foram Gram-positivos, catalase-negativos e, em sua maioria, pertencentes ao gênero Leuconostoc (73,3%). Em relação à resistência antimicrobiana, 96,6% dos isolados foram suscetíveis à ampicilina e 3,3% ao imipenem. Foi observada forte atividade inibitória contra S. aureus e E. coli, embora o sobrenadante livre de células não tenha apresentado efeito antagônico. A maioria das cepas (96,6%) sobreviveu em pH 2,5 e todas toleraram sais biliares. Elevada atividade proteolítica (>97%) foi detectada, enquanto a produção de diacetil, cápsula, lipase e peróxido de hidrogênio foram ausentes. A lactofermentação ocorreu em 96,6% das cepas, caracterizadas por formação de gel. Em conclusão, as BAL isoladas de queijo Minas Frescal apresentam características probióticas e tecnológicas promissoras, reforçando seu potencial de

aplicação em alimentos funcionais com benefícios à saúde intestinal e a segurança alimentar.

Palavras-chave: *Potencial probiótico. Alimentos funcionais. Atividade antimicrobiana. Leuconostoc. Produtos lácteos.*

1. Introduction

Fermentation with lactic acid bacteria (LAB) is considered one of the oldest methods of food preservation, as it produces a pH decrease and curbs the development of spoilage microorganisms and pathogens (Zapa´snik; Sokolowska; Bryla, 2022). The vast majority of starter cultures used in Brazil are still imported, which contributes to the costs associated with their production and reduces technological autonomy (Cabral et al., 2016). Therefore, the isolation and utilization of autochthonous LAB strains are a sustainable alternative for the economy, with the potential to provide added value from regional products and contribute to the development of functional foods specific to that area (Margalho et al., 2020).

LABs are Gram-positive, catalase-negative, and facultative anaerobic bacteria that produce lactic acid during carbohydrate fermentation (Giazzi et al., 2020). They can be divided into homofermentative and heterofermentative, and they include genera *Lactobacillus, Lactococcus, Leuconostoc, Streptococcus, Weissella, Pediococcus, Bifidobacterium*, and others (Zapa´snik; Sokolowska; Bryla, 2022). Apart from their technological function in acidification and the formulation of aroma and texture in fermented foods, LAB are of great interest to the industry due to their antimicrobial activity, which is connected with the production of organic acids, hydrogen peroxide, and bacteriocins (Giazzi et al., 2020; Zapa´snik; Sokolowska; Bryla, 2022).

A few LAB strains are also considered probiotics, providing health benefits such as modulation of the gut microbiota and a strengthened immune system (Das et al., 2022). The increased attention to probiotic species, such as *Lactobacillus* spp., and *Bifidobacterium* spp. has led to the investigation of isolates from traditional foods, including artisanal cheeses, that can play a dual role in inhibiting pathogens and improving sensorial quality (Vázquez-Lopez et al., 2019; Margalho et al., 2020).

Thus, the present study aimed to isolate and identify lactic acid bacteria from Minas Frescal cheese and evaluate their probiotic potential, as well as their technological properties.

2. Materials and Methods

2.1. Isolation of lactic acid bacteria

Two samples of Minas Frescal cheese were purchased from retail outlets in Cruz das Almas, Bahia, between June and July 2022. After collection, the samples were transported in thermal boxes to the Laboratory of Food and Environmental Microbiology (LABMAA) for immediate processing. For the isolation of lactic acid bacteria (LAB), 25 g of each sample was homogenized and plated on Man Rogosa Sharpe (MRS) agar, followed by incubation under anaerobic conditions at 37 °C for 48 h (Guimarães et al., 2018).

2.2. Biochemical characterization

The isolates were first subjected to morphological and biochemical characterization by Gram staining and the catalase test, in order to confirm the typical LAB profile (Gram-positive and catalase-negative). Subsequently, the isolates were

evaluated for gas production from glucose, arginine hydrolysis, growth at different temperatures (10 °C, 15 °C, and 45 °C), and growth in medium containing 6.5% NaCl, according to the identification key described by Schillinger and Lücke (1987).

2.3. Probiotic and technological characterization

The probiotic and technological properties of the isolates were assessed through the following assays: antimicrobial resistance (BrCast, 2017), antagonistic activity against *Staphylococcus aureus* and *Escherichia coli* (Mishra; Prasad, 2005), tolerance to different pH values and bile salt concentrations (Muñoz-Quezada et al., 2013), diacetyl production (Islam et al., 2021), lactofermentation capacity (Bramley; Mckinnon, 1990), hydrogen peroxide production (Samelis et al., 1994), capsule formation (Hitchener; Egan; Rogers, 1982), proteolytic activity (Phaff et al., 1994), and lipolytic activity (Leuschner et al., 1997).

4. Results and Discussion

The LAB count presented the results in 10^4 CFU/g in the two Minas Frescal cheese cultures, with 1.9×10^4 CFU/g, respectively, in Sample A and 2.4×10^4 CFU/g in Sample B. Similar results were reported by Boas et al. (2020), who attributed the low LAB populations to the use of commercial rennet instead of starter cultures intentionally added during processing.

In the 30 selected colonies, all of them showed typical LAB phenotypes (Gram-positive and catalase-negative). It was predominantly identified as genus *Leuconostoc* spp. according to biochemical identification (73.3%), followed by *Enterococcus* spp. (16.7%) and *Lactococcus* spp. (10%). In contrast, Margalho et al. (2020) also reported *Lactobacillus* as the dominant genus in artisanal cheese samples. These differences indicate the contribution of production size, sanitary procedures, ripening conditions, and local microbiota to driving LAB communities.

In the antimicrobial resistance test, all isolates were resistant to vancomycin, nalidixic acid, and imipenem, except for one isolate of *Leuconostoc* spp. strain, while the highest susceptibility was observed for ampicillin (Table 1). The indiscriminate use of antibiotics in human and veterinary medicine, along with horizontal gene transfer, contributes to this resistance profile (Nilo; Marin, 2022). Although concerning, resistance may favor probiotic strains by enabling their survival in the gastrointestinal tract during antimicrobial treatments (Islam et al., 2021). However, the presence of antimicrobial resistance genes in potential probiotic strains must be carefully evaluated, as these traits may be transferred to pathogenic bacteria, representing a public health concern. Thus, further genomic and functional studies are needed to ensure the safe application of resistant LAB as probiotics (Margalho et al., 2020).

Table 1. Antimicrobial susceptibility profile of lactic acid bacteria isolated from Minas Frescal cheese.

Antimicrobials	*Leuconostoc* spp. (22)			*Enterococcus* spp. (5)			*Lactococcus* spp. (3)		
	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)	S n (%)	I n (%)	R n (%)
Betalactâmicos									
Ampicilina	1 (5)	0	21 (96)	0	1 (20)	4 (80)	0	1 (33)	2 (67)
Imipenem	21 (96)	0	1 (5)	5 (100)	0	0	3 (100)	0	0
Glicopeptídeos									
Vancomicina	22 (100)	0	0	5 (100)	0	0	3 (100)	0	0
Quinolonas									
Ácido nalidíxico	22 (100)	0	0	5 (100)	0	0	3 (100)	0	0

(S) susceptible; (I) intermediate; (R) resistant; (n) number.

Antagonistic activity was significant, with all isolates exhibiting inhibition halos greater than 25 mm, and 53.3% showing stronger inhibition against *S. aureus* (Table 2). This effect is mainly attributed to bacteriocin production, which disrupts cell membranes and inhibits protein synthesis (Das et al., 2022; Garbacz, 2022), as well as the production of organic acids that lower pH and restrict microbial growth (Dias et al., 2018; Das et al., 2022; Garbacz, 2022).

Table 2. Inhibitory profile of lactic acid bacteria against indicator microorganisms.

Indicator microorganism	Zones of inhibition (mm)		
	Leuconostoc spp. (22)	*Enterococcus* spp. (5)	*Lactococcus* spp. (3)
Staphylococcus aureus ATCC 25922	22 (+++)	5 (+++)	3 (+++)
Escherichia coli ATCC 43300	22 (+++)	5 (+++)	3 (+++)

+ weak inhibition (up to 4 mm); ++ medium inhibition (5 to 9 mm); +++ strong inhibition (from 10 mm) (Chioda et al., 2007).

Regarding tolerance to acidic conditions, a gradual reduction in viability was observed as the pH decreased, with complete inhibition at a pH of 2.0 (Table 3). At pH levels of 3.0 and 2.5, 83.3% and 90% of the isolates, respectively, showed a reduction of one logarithmic cycle. Acid tolerance is essential for probiotic efficacy, since viable concentrations between 10^6 and 10^7 CFU/g are required to confer health benefits (Gonzalez et al., 2020; Ribeiro et al., 2020). According to Mishra and Prasad (2005), LAB strains tolerant to pH 3.0 are considered good probiotic candidates, suggesting that survival under acidic environments may be associated with their acidification capacity during fermentation (Giazzi et al., 2020; Gonçalves, 2021).

Table 3. Mean counts of lactic acid bacteria (log CFU/mL) exposed to different pH values.

Microorganisms (n = 30)	pH			
	7.0	3.0	2.5	2.0
Leuconostoc spp.	7.38 ± 0.04	6.57 ± 0.39	6.18 ± 1.41	-
Enterococcus spp.	7.37 ± 0.06	6.43 ± 0.01	6.46 ± 0.20	-
Lactococcus spp.	7.43 ± 0.01	6.74 ± 0.53	6.47 ± 0.12	-

Mean + standard deviation. *Leuconostoc* spp. (n = 22); *Enterococcus* spp. (n = 5); *Lactococcus* spp. (n = 3).

Lactococcus strains exhibited higher tolerance at pH 2.5 and 3.0 compared with the other isolates, whereas *Leuconostoc* strains showed the lowest resistance (Table 3). According to Muñoz-Quezada et al. (2013), LAB resistance varies greatly depending on the species and strain, which explains these differences.

In the bile salt tolerance assay, all strains maintained growth similar to the control up to 0.5%. At 0.7% concentration, a reduction of two logarithmic cycles was observed (Table 4). Despite this decrease, all isolates survived the concentrations tested, a key feature for survival in the gastrointestinal tract, as bile salts can destabilize cell membranes (Ribeiro et al., 2020).

Table 4. Mean counts of lactic acid bacteria (log CFU.mL^{-1}) exposed to different bile salt concentrations.

Microorganisms	**Bile salts**			
(n = 30)	**0%**	**0.3%**	**0.5%**	**0.7%**
Leuconostoc spp.	7.41 ± 0.03	7.37 ± 0.04	7.32 ± 0.04	5.49 ± 0.50
Enterococcus spp.	7.41 ± 0.03	7.35 ± 0.05	7.30 ± 0.05	5.25 ± 0.08
Lactococcus spp.	7.43 ± 0.03	7.33 ± 0.02	7.26 ± 0.03	5.25 ± 0.07

Mean + standard deviation. *Leuconostoc* spp. (n = 22); *Enterococcus* spp. (n = 5); *Lactococcus* spp. (n = 3).

Regarding metabolism, all strains were negative for diacetyl production. This compound, typical of heterofermentative strains, confers aromatic properties and antimicrobial activity in foods (Silva; Lopes; Bastos, 2022). The predominance of gelatinous (96.6%) and caseous (3.33%) lactofermentation profiles explains the absence of diacetyl, as this metabolic type is not associated with its synthesis.

None of the isolates produced hydrogen peroxide, indicating that the antagonistic effect observed was not due to this metabolite. Although hydrogen peroxide is commonly used as a food preservative for its bactericidal effect (Bordinassi et al., 2022; Abdelshafy et al., 2023), its absence does not compromise the probiotic potential of the strains. Capsule formation was also not detected, which is desirable since exopolysaccharides associated with capsule structures are linked to virulence (Forte et al., 2019; Gonçalves et al., 2022).

Enzymatic activity analysis revealed that 96.6% of isolates exhibited proteolytic activity, which is higher than the 86% reported by Carpiné et al. (2010), for positive strains. The high proteolytic activity observed is particularly relevant for cheese production, as it contributes to casein degradation, the release of bioactive peptides, and the development of desirable texture and flavor. This feature reinforces the technological potential of the isolates for designing of probiotic cheeses with distinct sensory attributes. On the other hand, no isolate showed lipolytic activity, consistent with Roma et al. (2020) and Islam et al. (2021). Low lipolytic activity is considered advantageous in cheeses, as it prevents undesirable flavors, despite the limited number of studies on LAB lipolysis.

These findings reinforce the importance of isolating LAB from dairy products such as cheeses, as they can provide strains with genetic variability and differentiated potential for technological and probiotic applications.

5. Conclusion

Lactic acid bacteria isolated from Minas Frescal cheese demonstrated promising probiotic and technological properties, including strong antagonistic activity against foodborne pathogens, tolerance to bile salts and acidic pH, and high proteolytic capacity. These characteristics are essential for their survival in the gastrointestinal tract and for their potential application in the development of functional dairy products. Importantly, the isolation of autochthonous strains from a widely consumed Brazilian cheese highlights both the scientific relevance and the potential for local innovation in the development of probiotic cultures. Thus, Minas Frescal cheese represents a valuable source of LAB with applications in food safety, gut health, and the formulation of functional foods.

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