UNIVERSIDADE FEDERAL DE LAVRAS

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LACTIC ACID BACTERIA ISOLATED FROM RAW MILK AND MINAS ARTISANAL CHEESE WITH ANTAGONISTIC POTENTIAL AGAINST PATHOGENS OF INTEREST IN THE DAIRY PRODUCTION CHAIN

LAVRAS – MG
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Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Graduate Program in Veterinary Sciences, to obtain the title of PhD.

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BACTÉRIAS ÁCIDO LÁTICAS ISOLADAS DE LEITE CRU E QUEIJO MINAS ARTESANAL COM POTENCIAL ANTAGÔNICO CONTRA PATÓGENOS DE INTERESSE NA CADEIA PRODUTIVA DO LEITE

Thesis presented to the Universidade Federal de Lavras, as part of the requirements of the Graduate Program in Veterinary Sciences, to obtain the title of PhD.

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Dedico à minha família.

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Nada na vida deve ser temido, somente compreendido. Agora é hora de compreender mais para temer menos.

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ABSTRACT

Lactic acid bacteria (LAB) have significant potential for application in the dairy production chain due to their ability to produce bioactive molecules with antimicrobial activity, as well as other characteristics of interest to the agri-food industry and health promotion. Samples of Minas artisanal cheese (MAC) (n = 59) from five traditional regions, and raw milk samples from 65 dairy herds in the Campo das Vertentes and South of Minas Gerais regions were used to prospect LAB searching for strains with antagonistic potential against pathogens of interest in the dairy production chain. From a total of 291 LAB isolates obtained from MAC, 84 strains were considered genetically distinct by REP-PCR and identified at the species level by MALDI-TOF. Among 352 presumptive LAB isolates from raw milk, 221 strains genetically distinct by rep-PCR were selected for species-level identification via MALDI-TOF. The antagonistic potential of LAB was tested using the spot-on-lawn technique, and the nature of the inhibitory substance was assessed through sensitivity to proteases and amylase. Strains obtained from MAC were tested against important pathogens and contaminants in MAC: *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 5779, and *Escherichia coli* O157 IAL 1848, whereas LAB isolated from raw milk were evaluated against pathogens causing bovine mastitis: *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *E. coli* O157 IAL 1848, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus uberis* ATCC 700407, and *E. faecalis* ATCC 29212. Susceptibility to antimicrobials: ampicillin, chloramphenicol, cotrimoxazole, erythromycin, streptomycin, gentamicin, penicillin G, tetracycline, and vancomycin was assessed by disk diffusion on agar. Genes encoding antimicrobial resistance (*aac*A-*aph*D, *erm*A/B, *tet*M/O/K/L/S, *bla*Z, and *van*A/B) were investigated via PCR. The results revealed three superior strains of the genus *Lactobacillus* spp. (codes 52, 177, and 272G) from MAC, susceptible to antibiotics and bioprotective against *L. monocytogenes* and *E. coli in vitro* and in coculture in milk. Among the LAB strains isolated from raw milk, the *Lactococcus lactis* CV151 stood out for its strong antagonistic activity against all the tested mastitis pathogens, being susceptible to all the antibiotics and showing no resistance genes; *Enterococcus faecium* CV167 exhibited antagonistic activity against most of the tested pathogens, with three bacteriocin-related genomic regions identified via genomic analyses; and *Weissella cibaria* CV04 demonstrated excellent antagonistic activity against all the tested mastitis pathogens. These results contribute to advancing the knowledge of the LAB present in milk and MAC, paving the way for applications in agribusiness and animal health.

Keywords: antibiotic resistance; bioprotection; mastitis; probiotics.

RESUMO

As bactérias ácido láticas (BAL) apresentam grande potencial de aplicação na cadeia produtiva do leite devido a sua habilidade em produzir moléculas bioativas, que podem apresentar atividade antimicrobiana, bem como outras características de interesse para a agroindústria e na promoção da saúde. Amostras de Queijo Minas Artesanal (QMA) (*n*=59) provenientes de cinco regiões tradicionais e amostras de leite cru de 65 rebanhos leiteiros bovinos das regiões Campo das Vertentes e Sul de Minas Gerais foram utilizadas para prospectar BAL em busca de linhagens com potencial antagônico contra patógenos de interesse na cadeia produtiva do leite. De um total de 291 isolados de BAL obtidos de QMA, 84 linhagens foram consideradas geneticamente distintas por rep-PCR e identificadas em nível de espécie por MALDI-TOF. De 352 bactérias presuntivamente classificadas como BAL isoladas de leite cru, 221 linhagens geneticamente distintas, por rep-PCR, foram selecionadas para identificação a nível de espécie por MALDI-TOF. O potencial antagônico das BAL foi testado através da técnica *spot-on-lawn* e a natureza da substância inibitória foi avaliada através da sensibilidade a proteases e amilase. As linhagens obtidas de QMA foram testadas contra os patógenos e contaminantes importantes em QMA: *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 5779 e *Escherichia coli* O157:H7 IAL 1848, enquanto que as BAL isoladas de leite cru foram avaliadas contra os patógenos causadores da mastite bovina: *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *E. coli* O157:H7 IAL 1848, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus uberis* ATCC 700407 e *E. faecalis* ATCC 29212. A avaliação da suscetibilidade a nove antimicrobianos foi realizada por disco de difusão em ágar. Os genes que codificam para resistência a antimicrobianos *aac*A-*aph*D, *erm*A/B, *tet*M/O/K/L/S, *bla*Z, *van*A/B foram pesquisados por PCR. Os resultados apontaram três linhagens superiores do gênero *Lactobacillus* spp. (códigos 52, 177 e 272G), oriundas de QMA, suscetíveis a antibióticos e bioprotetoras contra *L. monocytogenes* e *E. coli in vitro* e em co-cultivo no leite. Das BAL isoladas de leite cru, destacam-se a linhagem *Lactococcus. lactis* CV151 por sua boa atividade antagônica contra todos os patógenos da mastite testados e suscetibilidade aos antimicrobianos; *Enterococcus faecium* CV167 que apresentou atividade antagônica contra a maioria dos patógenos testados e com três regiões do genoma relacionadas a produção de bacteriocinas; e *Weissella cibaria* CV04 por sua excelente atividade antagônica contra todos os patógenos da mastite testados. Estes resultados contribuem para o avanço do conhecimento de BAL em leite e QMA, abrindo caminho para aplicações na agroindústria e sanidade animal.

Palavras-chave: bioproteção; mastite; probióticos; resistência a antibióticos.

IMPACT INDICATORS

The research demonstrates significant social, technological, economic, and cultural impacts by highlighting the potential applications of lactic acid bacteria (LAB) in the dairy production chain. By identifying strains with high antimicrobial potential against key pathogens such as *Listeria monocytogenes*, *Escherichia coli*, and *Staphylococcus aureus*, the study directly contributes to food safety, animal health, and public health. The innovative application of LAB and their metabolites in controlling livestock diseases such as mastitis provides effective alternatives to reducing antibiotic use, mitigating antimicrobial resistance, and decreasing the contamination of food and the environment with antibiotic residues. The isolation of LAB from Minas artisanal cheese (MAC), with potential for bioprotection and the preservation of dairy products, enhances the value of this cultural and economic heritage of Minas Gerais, while enabling the development of safer and higher-quality artisanal dairy products for consumers. The significance of this work is further reflected in its extensionist nature, highlighting potential partnerships with dairy and MAC producers, strengthening sustainable practices, and disseminating results to local communities. The research directly impacts population groups in Minas Gerais, including producers, technicians, partner institutions, and QMA consumers, benefiting the global dairy production chain. Classified within the thematic areas of health and technology and production, the study contributes to various United Nations Sustainable Development Goals, such as Good Health and Well-Being, Industry, Innovation and Infrastructure, and Responsible Consumption and Production. The results promote safer and more sustainable practices in dairy production, delivering significant benefits to animal and human health while driving advancements in the agro-industrial sector and reaffirming the transformative role of science in addressing global challenges.

INDICADORES DE IMPACTO

A pesquisa apresenta impactos relevantes nos âmbitos social, tecnológico, econômico e cultural, ao demonstrar o potencial de aplicação das bactérias ácido láticas (BAL) na cadeia produtiva do leite. Ao identificar linhagens com elevado potencial antimicrobiano contra patógenos de destaque, como *Listeria monocytogenes*, *Escherichia coli* e *Staphylococcus aureus*, o estudo contribui diretamente para a segurança alimentar, a sanidade animal e a saúde pública. A aplicação inovadora de BAL e seus metabólitos no controle de doenças em rebanhos, como a mastite, oferece alternativas eficazes à redução do uso de antibióticos, mitigando a resistência antimicrobiana e diminuindo a contaminação de alimentos e do meio ambiente por resíduos desses medicamentos. O isolamento de BAL de Queijo Minas Artesanal (QMA), com potencial para bioproteção e conservação de produtos lácteos, valoriza esse patrimônio cultural e econômico de Minas Gerais, além de possibilitar o desenvolvimento de produtos lácteos artesanais autorais mais seguros e de alta qualidade para os consumidores. A relevância do trabalho se reflete ainda no caráter extensionista, evidenciando a potencial parceria com produtores de leite e de QMA, fortalecendo práticas sustentáveis e disseminando os resultados para comunidades locais. O impacto atinge diretamente grupos populacionais do estado de Minas Gerais, incluindo produtores, técnicos, instituições parceiras e consumidores de QMA, beneficiando toda a cadeia produtiva do leite global. Classificado nas áreas temáticas de saúde e tecnologia e produção, o trabalho contribui para diversos Objetivos de Desenvolvimento Sustentável da ONU, como saúde e bem-estar, inovação industrial e consumo responsável. Os resultados apresentados promovem práticas mais seguras e sustentáveis na produção láctea, gerando benefícios significativos para a sanidade animal e humana, além de avanços no setor agroindustrial, reafirmando o papel transformador da ciência na superação de desafios globais.

SUMMARY

GENERAL INTRODUCTION

Minas Gerais stands out in Brazil as the largest producer of milk and artisanal cheeses, with Minas Artisanal Cheese (MAC) representing a century-old tradition (KAMIMURA et al., 2019). MAC holds significant cultural value and is renowned for its unique quality and flavor, which results from traditional production methods that preserve specific microbiological and sensory characteristics (NERO et al., 2021). However, the use of raw milk in the manufacturing process makes these products susceptible to microbiological contamination, posing serious health risks to consumers. Among the main pathogens that can be transmitted through artisanal cheeses, *Listeria monocytogenes, Escherichia coli*, *Staphylococcus aureus, Brucella abortus* and *Mycobacterium bovis* stand out (CARNEIRO AGUIAR et al., 2022; OLIVEIRA et al., 2018; SILVA et al., 2022). Therefore, ensuring microbiological safety is essential for maintaining the quality and safety of these traditional products.

The dairy production chain faces continuous challenges in the search for effective methods that ensure the quality and safety of dairy products while promoting the health of dairy herds. Among the most pressing challenges are the risks associated with bovine mastitis, a highly prevalent disease in dairy herds that affects both milk production and quality, and may facilitate the contamination of disease-causing pathogens in dairy products. Mastitis not only impacts animal productivity by reducing milk volume and altering its composition, but also increases operational costs due to the treatment of affected animals and the need to discard milk contaminated with antimicrobials (RODRIGUEZ et al., 2024). Furthermore, the extensive use of antibiotics to control this disease has raised concerns about bacterial resistance and the presence of drug residues in milk, as mastitis is the primary cause of antibiotic use on dairy farms (DE CAMPOS et al., 2021; RODRIGUEZ et al., 2024; RUEGG, 2018).

Given the significance of these challenges, the search for effective methods to control mastitis in herds, as an alternative and/or adjuvant to antimicrobials, as well as tools that increase the safety of artisanal dairy products, is crucial to maintaining the economic viability of production systems. One promising approach to mitigate the risks associated with pathogens in the dairy production chain is the use of lactic acid bacteria (LAB) with bioprotective and probiotic properties (FAVARO et al., 2014; UYENO et al, 2015). These bacteria can be isolated from natural sources, such as raw milk and MAC, and are widely recognized for their ability to inhibit the growth of undesirable microorganisms through the production of antimicrobial substances, including lactic acid, hydrogen peroxide, and bacteriocins (HERNÁNDEZ

FIGUEROA; LÓPEZ-MALO; MANI-LÓPEZ, 2024). The use of LAB and/or their metabolites in the dairy chain can promote animal health and food safety without the adverse effects associated with traditional methods. Moreover, LAB play a fundamental role in fermentation and the enhancement of sensory characteristics in artisanal cheeses and other dairy products (PARVEZ et al., 2006).

LAB constitute a diverse group of microorganisms widely distributed in nature and are commonly associated with low pH environments, such as the gastrointestinal tract of animals, plants, soil, and fermented products (WANG et al., 2021). These bacteria are characterized as Gram-positive rods or cocci, catalase-negative and oxidase-negative, facultative anaerobes, and non-spore-forming, producing lactic acid as the main metabolic product of carbohydrate fermentation (MGOMI et al., 2023; MORA-VILLALOBOS et al., 2020). Their importance in the food industry is related to their carbohydrate fermentation capacity, production of lactic acid, and secondary metabolites, which confer unique organoleptic characteristics and preservative properties to foods (AYIVI et al., 2020; MOKOENA; OMATOLA; OLANIRAN, 2021).

Most LAB belong to the phylum Firmicutes, class Bacilli, and order Lactobacillales, which includes six families: *Aerococcaceae, Carnobacteriaceae, Enterococcaceae, Lactobacillaceae*, *Leuconostocaceae*, and *Streptococcaceae*, with more than 60 genera and 300 species, a number that continues to grow as new discoveries are made (MORA-VILLALOBOS et al., 2020; WANG et al., 2021). The classification of LAB into different groups is based on various characteristics, according to the criteria originally established by Orla-Jensen in 1919, such as cell morphology, mode of glucose fermentation, ability to utilize sugars, growth at different temperatures, adaptation to nutrient-rich environments for lactic acid production, ability to grow in high salt concentrations (NaCl), and tolerance to acidic or alkaline conditions (NASR; ABD-ALHALIM, 2024). This classification system initially recognized only four LAB genera: *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* (AYIVI et al., 2020).

With the advancement of molecular tools and genetic characterization, the nomenclature of LAB has been reorganized, and new groups have been identified (ZHENG et al., 2020). The molecular characterization of these bacteria involves advanced techniques such as random amplified polymorphic DNA profiling, 16S rRNA gene sequencing, PCR-based fingerprinting, and soluble protein pattern analysis (SHARMA; PARK, 2020). These advancements enable a more precise and detailed classification of LAB, facilitating the identification of strains with specific properties relevant for industrial and health applications. Additionally, whole-genome sequencing (WGS) has allowed for in-depth analysis of metabolic pathways, probiotic potential, safety, and antimicrobial compound production capabilities (SHARMA; PARK, 2020; YE; LI; GU, 2020), opening new perspectives for the use of these bacteria in functional food formulations and in the biological control of pathogens.

Based on this advancement in molecular technologies, a publication in the *International Journal of Systematic and Evolutionary Microbiology* in April 2020 revealed the recent nomenclature for the genera *Lactobacillus* and *Leuconostoc* (ZHENG et al., 2020). In addition to the merging of Lactobacillaceae and Leuconostocaceae into one family, Lactobacillaceae, the genus *Lactobacillus* was reclassified by scientists into 25 genera: *Lactobacillus*, *Paralactobacillus*, *Amylolactobacillus*, *Acetilactobacillus*, *Agrilactobacillus*, *Apilactobacillus*, *Bombilactobacillus*, *Companilactobacillus*, *Dellaglioa*, *Fructilactobacillus*, *Furfurilactobacillus*, *Holzapfelia*, *Lacticaseibacillus*, *Lactiplantibacillus*, *Lapidilactobacillus*, *Latilactobacillus*, *Lentilactobacillus*, *Levilactobacillus*, *Ligilactobacillus*, *Limosilactobacillus*, *Liquorilactobacillus*, *Loigolactobacillus*, *Paucilactobacillus*, *Schleiferilactobacillus*, and *Secundilactobacillus*.

Each species of LAB has distinct metabolic characteristics that influence its role in food fermentation and its ability to interact with other microorganisms, including pathogens (AYIVI et al., 2020). LAB are facultative anaerobes that metabolize sugars through fermentation, producing mainly lactic acid as the final product. These bacteria utilize carbohydrates as sources of carbon and energy without the use of oxygen and produce peroxidases to protect against damage caused by oxygen byproducts. Additionally, LAB can produce a variety of metabolites, such as organic acids and bacteriocins, which confer antimicrobial properties (MOKOENA; OMATOLA; OLANIRAN, 2021).

The metabolism of LAB can involve two main pathways: homofermentative and heterofermentative. Homofermentation involves the metabolism of disaccharides by these bacteria into lactic acid, whereas heterofermentation results in the formation of ethanol, carbon dioxide, hydrogen peroxide, diacetyl, acetoin, and acetaldehyde, in addition to lactic acid (MOKOENA; OMATOLA; OLANIRAN, 2021). The most commonly used homofermentative LAB in the food chain are from the genera *Lactobacillus*, *Lactococcus*, and *Streptococcus*, which produce two molecules of lactate from one molecule of glucose (AYIVI et al., 2020). Homofermentative bacteria can also be classified on the basis of their growth temperature as mesophilic or thermophilic. Mesophilic LAB grow optimally at approximately 30 °C, while thermophilic LAB grow optimally at 42 °C. The main heterofermentative LAB belong to the genera *Weissella*, *Leuconostoc*, and some *Lactobacillus* species, which generate lactate, ethanol, and carbon dioxide from one molecule of glucose (AYIVI et al., 2020).

LAB are generally recognized as safe (GRAS) and are thus widely used in food products and probiotics (MATHUR; BERESFORD; COTTER, 2020). Several studies have highlighted the significant potential of LAB as bioprotective agents in the dairy industry because of their ability to inhibit the growth of important pathogens and spoilage microorganisms (BEN SAID et al., 2019; CROWLEY; MAHONY, 2013; FAVARO; BARRETTO PENNA; TODOROV, 2015; GÓMEZ et al., 2016; RENDUELES et al., 2022; SOUZA et al., 2022). Moreover, research has demonstrated that LAB isolated directly from milk and dairy products have strong bioprotective potential against *L. monocytogenes* and *E. coli* (AGUILAR; VANEGAS; KLOTZ, 2011; GÓMEZ et al., 2016; PANEBIANCO et al., 2021), two major pathogens of concern in the dairy industry. The identification and isolation of LAB from MAC can contribute to the development of starter or adjunct cultures capable of inhibiting pathogen growth and thereby improving the safety and quality of artisanal cheeses.

Some LAB species exhibit probiotic characteristics, meaning that they can confer health benefits when consumed in adequate quantities (HILL et al., 2014). In terms of animal health, these benefits include improved gastrointestinal health, strengthened immune systems, and prevention of diseases. Several studies have encouraged the use of LAB in the control of bovine mastitis (ALHARBI; ALSALOOM, 2021; BENNETT; FLISS; MALOUIN, 2022; BROADBENT et al., 1989; CAO et al., 2007; CRISPIE et al., 2008; KLOSTERMANN et al., 2008). As previously mentioned, the bioprotective and probiotic properties of LAB are related to their production of antimicrobial metabolites, including organic acids, ethanol, hydrogen peroxide, diacetyl, CO2, and bacteriocins (HOSKEN et al., 2023).

Bacteriocins are small, ribosomally synthesized antimicrobial peptides produced by bacteria, such as LAB (HAMMAMI; FERNANDEZ, 2013; HERNÁNDEZ-GONZÁLEZ et al., 2021). These peptides can exhibit antimicrobial effects against a wide range of microorganisms, including bacteria, fungi, parasites, viruses, and naturally resistant structures such as bacterial biofilms (HERNÁNDEZ-GONZÁLEZ et al., 2021). The potential of bacteriocins in controlling mastitis is particularly promising, as these molecules not only inhibit pathogens known to cause the disease (KITAZAKI et al., 2017), but also target pathogenic microorganisms that form biofilms in the mammary gland (TITZE; KRÖMKER, 2020), which are difficult to eliminate using conventional methods. Furthermore, bacteriocins have strong technological appeal due to their antimicrobial efficacy combined with a low likelihood of inducing bacterial resistance, owing to their multiple or specific mechanisms of action (HERNÁNDEZ-GONZÁLEZ et al., 2021).

The isolation of LAB from natural sources, such as raw milk and artisanal cheeses, has been extensively investigated, as these bacteria often display more robust activity when sourced from the same ecological niches (SAARELA et al., 2000). These microorganisms develop genetic and physiological adaptations that reflect the complexity of their environments, resulting in unique characteristics that can be harnessed for various biotechnological applications. The specific conditions of these natural environments promote the selection of strains with special properties, such as resistance to adverse conditions and production of bioactive compounds (MALDONADO et al., 2012).

Given this context, the present study aimed to isolate and characterize LAB from raw milk and MAC, evaluating their bioprotective and probiotic potential against important pathogens associated with the dairy production chain. The characterization of these LAB included species identification, their ability to inhibit pathogen growth both *in vitro* and in milk, their susceptibility to antimicrobials, and the determination of probiotic properties.

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CHAPTER – 1: Necessary requirements for a bacterial strain to be considered potentially probiotic

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ABSTRACT

Probiotics are live microorganisms that, when administered in adequate amounts, confer health benefits to the host and are safe for their intended use. To classify a bacterial strain as potentially probiotic, it must meet strict criteria involving safety, functionality, and efficacy. Regulatory guidelines vary globally, with authorities such as the European Food Safety Authority (EFSA), the Food and Drug Administration (FDA), and, in Brazil, the National Health Surveillance Agency (ANVISA), establishing requirements based on scientific evidence. This article discusses the essential requisites for bacterial strains to be considered probiotics, emphasizing safety, gastrointestinal survival or survival in the target environment, such as in the mammary gland, epithelial adhesion, and functional efficacy. Safety assessments ensure the absence of transferable antibiotic resistance genes, toxins, and virulence factors. Furthermore, probiotic efficacy must be validated through *in vitro, in vivo*, and clinical studies, demonstrating benefits such as pathogen antagonism, immune modulation, and health maintenance. The guidelines outlined by ANVISA provide a structured approach to evaluating probiotic strains, incorporating taxonomic identification, safety tests, and functional assays. These criteria aim to ensure that probiotics used in food or supplements are effective and safe for consumers. This review highlights the complexity of selecting and validating potential probiotic strains, contributing to the development of functional foods and supplements with proven health benefits.

Keywords: bioprotection, efficacy, functionality, safety, regulatory guidelines.

1. INTRODUCTION

Probiotics are defined as live microorganisms that when administered in adequate amounts confer health benefits to the host and are safe for their intended use (HILL et al., 2014). Probiotic microorganisms belong to various genera and species, including both bacteria and yeasts, and have been associated with numerous beneficial effects on human and animal health (SHOKRYAZDAN et al., 2017). For a bacterial strain to be considered potentially probiotic, several requirements and criteria must be met, encompassing aspects of safety, functionality, and efficacy. Defining these criteria has been a fundamental goal for various stakeholders in the field of probiotics (BINDA et al., 2020).

Each country or region regulates these criteria through regulatory agencies, such as the European Food Safety Authority (EFSA) in Europe and the Food and Drug Administration (FDA) in the United States, based on scientific evidence of safety and efficacy (BINDA et al., 2020; DI GIOIA; BIAVATI, 2018). In Brazil, the National Health Surveillance Agency (Agência Nacional de Vigilância Sanitária - ANVISA) oversees the regulation and evaluation of probiotics for use in foods, according to the requirements of Resolution RDC No. 241, dated July 26, 2018 (BRAZIL, 2018). Guidelines and recommendations developed by foreign regulatory authorities and experts have served as important references for drafting Brazilian regulations, drawing from documents tailored to probiotics (HILL et al., 2014).

The evaluation of a probiotic strain by ANVISA involves three essential components: unequivocal confirmation of the strain's identity, assessment of its safety, and demonstration of its beneficial effects (Chart 1) (ANVISA, 2021).

Chart 1. Steps for the evaluation of a probiotic strain for consumption by ANVISA.

<table>
<tr><td rowspan="6">Phase 1</td><td rowspan="6">Identity confirmation</td><td colspan="3">Nomenclature</td></tr>
<tr><td colspan="3">Deposit in culture collection</td></tr>
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<tr><td rowspan="2">Identification</td><td colspan="2">Phenotypic tests</td></tr>
<tr><td colspan="2">Genotypic tests</td></tr>
<tr><td colspan="3">Manufacturing process</td></tr>
<tr><td rowspan="11">Phase 2</td><td rowspan="11">Proof of safety</td><td colspan="3">Identification of the microorganism's risk group or class</td></tr>
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<tr><td rowspan="3">Criteria for study selection</td><td>Target population</td></tr>
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</table>

National Health Surveillance Agency (ANVISA, 2021).

Regardless of the regulations established by each regulatory entity, Binda et al. (2020) emphasized the importance of clearly and meticulously defining the criteria that determine the probiotic potential of microbial strains. This process should utilize decision trees (Figure 1) and provide detailed guidance on how to meet these criteria without delving into the specifics of potential mechanistic requirements. The authors further outline the key requirements that probiotic strains must meet for inclusion in foods or supplements intended for human consumption, as follows (BINDA et al., 2020):

a) Be sufficiently characterized;
b) Be safe for the intended use;
c) At least one positive human clinical trial conducted according to generally accepted scientific standards;
d) A sufficient number of viable bacteria remain in the product at an effective dose throughout the entire shelf-life.

The following sections discuss the main aspects related to these criteria that determine the probiotic potential of bacterial strains, on the basis of the *Guidelines for the Procedural Submission of Probiotic Evaluation Petitions for Use in Food* by ANVISA (ANVISA, 2021).

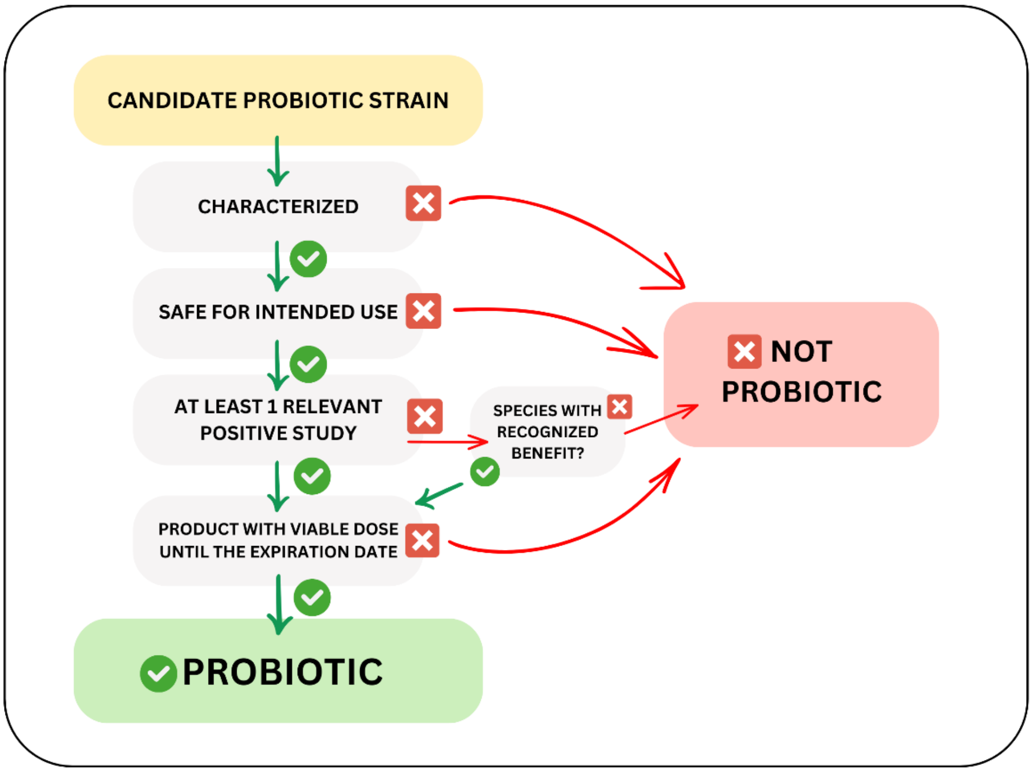

Figure 1. Decision tree for determining whether a probiotic candidate meets the defined criteria. Adapted from Binda et al. (2020).

1. Identity confirmation

Accurate identification of a bacterial strain is the initial step in evaluating its probiotic attributes (THAKUR; ROKANA; PANWAR, 2016). Proper designation of a strain involves two primary components: the official names of the genus and species (and subspecies, if applicable) following nomenclature rules, and the strain designation, which may be either the catalog number from a recognized culture collection or a commercial identifier (BINDA et al., 2020). This classification enables the prediction of the strain's general properties based on existing knowledge about its taxonomic group. It is also critical for risk identification, as it provides a reference for relevant characteristics, such as the potential to produce specific toxins, allergens, or virulence factors typically expressed by the genus or species (ANVISA, 2021).

The characterization of a potentially probiotic bacterial strain is crucial for safety evaluation because the observed properties, including specific virulence factors and resistance traits, are strain-specific (JACOBSEN et al., 1999; SHOKRYAZDAN et al., 2017). Strain identity is also essential for associating microorganism with functional and beneficial effects, supporting their probiotic activity (BINDA et al., 2020). Information about the strain's origin, including its isolation source—such as food, the human or animal microbiota, or other potential sources—should be well-documented. This is important because the probiotic effects of microorganisms are host-specific (SAARELA et al., 2000), and their effectiveness tends to be greater when strains are derived from their natural habitat (UYENO; SHIGEMORI; SHIMOSATO, 2015).

Most probiotic bacteria used for human and animal applications belong to the lactic acid bacteria (LAB) group (SHOKRYAZDAN et al., 2017). Historically, various phenotypic characteristics of LAB, such as morphological, physiological, and biochemical traits, have been employed to differentiate and identify species (OCHMAN; LERAT; DAUBIN, 2005; SHOKRYAZDAN et al., 2017). However, phenotypic methods, including morphological analysis and Gram staining, are recommended only for the initial screening of isolates, as these techniques alone are insufficient for proper identification (BINDA et al., 2020).

A range of molecular methods are currently available for bacterial species identification and strain differentiation. These methods include 16S rDNA gene sequencing, matrix-assisted laser desorption/ionization mass spectrometry (MALDI-TOF), and pulsed-field gel electrophoresis (PFGE) (NACEF et al., 2017; SHOKRYAZDAN et al., 2017). Nevertheless, the current "gold standard" for identifying probiotic bacterial strains is whole-genome sequencing (WGS), which enables robust and precise identification of specific strains (BINDA et al., 2020). Furthermore, WGS facilitates the detection of risk factors, such as virulence and antimicrobial resistance genes, as well as genes associated with probiotic activity. It also allows the identification of potential plasmids commonly found in some LAB, which may play an important role in probiotic functionality (BINDA et al., 2020).

2. Verification of safety

A probiotic strain must be safe for its intended use, taking into account the target population and the recommended usage conditions. Safety must be demonstrated through *in vitro* and *in vivo* tests capable of confirming the strain's harmlessness (ANVISA, 2021). Additionally, historical data on safe use can play a significant role in the overall safety evaluation of a probiotic strain for its intended application (BINDA et al., 2020).

The first step in assessing a strain's safety is identifying the risk group or classification to which the bacteria belong, along with its reference. For instance, LAB strains isolated from food and fermented products are generally recognized as safe (GRAS) and are widely studied for their health benefits (MATHUR; BERESFORD; COTTER, 2020). However, these safety characteristics cannot be extrapolated to other strains without prior investigation (THAKUR; ROKANA; PANWAR, 2016). The safety of each strain is evaluated individually, and no specific safety criteria can be generalized to favor approval (BINDA et al., 2020).

A probiotic strain should have a history of safe use in foods or dietary supplements. This can be demonstrated through epidemiological data and scientific reviews (SALMINEN et al., 1998). Safety based on a history of safe use requires evidence that the strain has been consumed for generations, on a large scale, and by a genetically diverse group of individuals without reports of adverse events. Such evidence is particularly important for products with a well-documented tradition of use in other countries, provided that the current purpose and conditions of use align with those historically described (ANVISA, 2021).

2.1 *In vitro* assays

The necessity of conducting *in vitro* assays depends on the availability of information regarding the characteristics or properties of the strain's taxonomic unit, its history of use, the target population, and, when applicable, findings from animal and human studies. At a minimum, these assays must confirm that microorganisms lack specific antibiotic resistance genes with horizontal transfer potential and that any virulence factors that could pose risks to consumer health. Additional tests may be needed to evaluate traits such as the production of antimicrobial substances, lactate, mucinase, and bile salt deconjugation, among others (ANVISA, 2021).

2.1.1 Antimicrobial resistance profile of clinical relevance

This assay is mandatory for all probiotic strains (BRAZIL, 2018). Antimicrobial resistance (AMR) is a growing global health concern (TUFA et al., 2023). With respect to probiotic bacteria, the primary concern is the presence of antibiotic resistance genes and their potential transfer to cohabitant microorganisms via mobile genetic elements (DANIALI; NIKFAR; ABDOLLAHI, 2020).

The development of AMR occurs through various mechanisms. While horizontal gene transfer is likely the principal mechanism for resistance dissemination among probiotic strains—facilitating gene exchange between bacteria in the same environment (MANTEGAZZA et al., 2018)—chromosomal mutations may also confer resistance to antibiotics, such as vancomycin (DANIALI; NIKFAR; ABDOLLAHI, 2020). However, resistance arising from chromosomal mutations is rarely transmitted through mobile genetic elements. Intrinsic resistance, inherent to certain species, is also widely observed in LAB (GUEIMONDE et al., 2013).

Intrinsic resistance is presumed to have minimal potential for horizontal transference. In contrast, resistance mediated by genes located on mobile genetic elements is considered to have high potential for horizontal spread (LI et al., 2020). Therefore, all potentially probiotic bacterial strains must be evaluated for susceptibility to a relevant panel of antimicrobials critical for human and veterinary medicine. If resistance to any antibiotic is detected, it is crucial to determine whether it is intrinsic or acquired. Furthermore, among the acquired resistance, it is essential to differentiate between resistance caused by random genetic mutations in the microbial genome and that resulting from the acquisition of mobile genetic elements (ANVISA, 2021).

To assess antimicrobial susceptibility, serial dilution techniques in agar or broth must be employed, incorporating control strains into the tests. A basic requirement is to determine the minimum inhibitory concentration (MIC) of clinically relevant antibiotics, expressed in mg/L or µg/mL. Qualitative or semiqualitative methods, such as diffusion techniques, are generally not acceptable. Moreover, ANVISA regulations stablish that probiotics intended for human use must be susceptible to at least two clinically significant antibiotics to ensure that effective treatment options are available in the event of infections (ANVISA, 2021).

2.1.2 Investigation of virulence factors

Virulence factors are another critical aspect of strain-level evaluation for safety, including the capacity to produce toxins, enzymes (e.g., hemolysins and mucinases), toxic metabolites (e.g., biogenic amines), D-lactate, or other pathogenicity-related molecules (e.g., invasins, adhesins, and surface proteins). These characteristics can often be assessed through genome analysis, when feasible, or through standardized phenotypic tests (BINDA et al., 2020).

2.1.3 Complementary tests

The production of antimicrobial substances, as determined through *in vitro* studies against pathogens, plays a key role in defining a bacterial species as probiotic. These substances include bacteriocins, organic acids, hydrogen peroxide, diacetyl, acetaldehyde, acetoin, carbon dioxide, reuterin, reutericyclin, and other compounds (SHOKRYAZDAN et al., 2017).

Additionally, a complementary analysis must address potential adverse effects on the host's beneficial microbiota that might result from these antimicrobial properties (ANVISA, 2021). Probiotics must not negatively impact commensal microorganisms in the human or animal microbiota in a way that could harm host health (CLEUSIX et al., 2007).

2.2 *In vivo* assays

Animal models and clinical studies are essential for assessing the safety of probiotic strains, including the absence of adverse effects such as toxicity or infections (ISHIBASHI; YAMAZAKI, 2001). These studies must include evaluations of genotoxicity/mutagenicity, acute toxicity, subchronic toxicity, long-term toxicity, and reproductive and developmental toxicity (ANVISA, 2021). Beyond the standard endpoints of developmental toxicity testing, it is crucial to investigate whether a probiotic strain could become a permanent resident in the adult offspring microbiota (SANDERS et al., 2010).

Traditional safety assessments, which focus on determining the toxicity or pathogenicity of probiotic bacteria, have inherent limitations. Simplified animal models or cell-based assays cannot fully replicate the complex interactions between microorganisms, their environment, and human populations (ANVISA, 2021). Human intervention studies, on the other hand, provide robust documentation of the safety and tolerance of probiotics through rigorous monitoring and reporting of adverse events. Biological and clinical parameters, including vital signs, can be tracked to gather critical safety data. Unexpected deviations from baseline values or patterns may indicate safety concerns. Importantly, any study, particularly those of longer duration or involving larger sample sizes, will inevitably report adverse events. The key factor is determining whether adverse events differ between intervention groups (e.g., probiotics and placebo) and/or if they are causally linked to the intervention (BINDA et al., 2020).

3. Functionality

To be considered potentially probiotic, bacterial strains must exhibit specific properties. In addition to accurate identification and safety aspects, other characteristics need to be defined, as probiotic properties are strain-specific and influenced by cultivation conditions and dosage. It is unlikely that two strains of the same species will exhibit identical probiotic characteristics. Therefore, only well-defined strains should be used as probiotics (SHOKRYAZDAN et al., 2017).

The following are the primary tests commonly used for screening and characterizing potential probiotic microorganisms, as recommended by the Food and Agriculture Organization/World Health Organization (FAO/WHO, 2002):

- Resistance to gastric acidity;
- Bile salt hydrolase (BSH) activity and bile salt resistance;
- Adhesion to mucus and/or human epithelial cells or cell lines;
- Antimicrobial activity and antagonism against potentially pathogenic bacteria.

3.1 Survival and viability in the gastrointestinal tract

To be effective, a probiotic strain must withstand adverse conditions of the gastrointestinal tract (GIT), including the acidic pH of the stomach and the presence of bile considering its oral use. Both *in vitro* and *in vivo* assays can assess this resistance.

Any probiotic administered orally to humans or animals must be acid- and bile-tolerant due to the host's stressful GIT environment, which includes gastric acids and bile salts (SHOKRYAZDAN et al., 2017). Stress in the human GIT begins in the stomach, where transit time can range from less than 1 hour to 3–4 hours, depending on individual factors, diet, and other conditions (RUIZ-MOYANO et al., 2008). The gastric pH can decrease to 1.5, whereas bile concentrations vary in the upper intestinal tract (SHOKRYAZDAN et al., 2017). Thus, acid and bile tolerance is a fundamental criterion when selecting a probiotic strain for oral administration.

In addition to survival and colonization, tolerance to GIT stress is critical for maintaining the probiotic strain’s cellular integrity, adhesion capability, and metabolic activity (SHOKRYAZDAN et al., 2017). *In vitro* results on acid and bile tolerance often predict a strain's survival capability in the host. However, this attribute is relevant only for oral applications and may not apply to other probiotic uses. Conjugated bile salts are known to inhibit the growth of both Gram-negative and Gram-positive bacteria, with Gram-positive strains generally being more sensitive. Some probiotic bacterial strains can produce bile salt hydrolase (BSH), encoded by the *bsh* gene, which hydrolyzes bile salts and is associated with their resistance to these compounds (SHOKRYAZDAN et al., 2017).

3.2 Adherence to the intestinal epithelium

Adherence to the intestinal epithelium is vital for colonization and interaction with the host immune system, making it one of the primary criteria for selecting probiotic bacterial strains (KRAUSOVA; HYRSLOVA; HYNSTOVA, 2019). Bacterial cell wall hydrophobicity plays a fundamental role in bacterial adhesion. Hydrophobic interactions form immediately upon contact and can strengthen over time as water is removed from between the surfaces (ESPECHE et al., 2009; YOUNES et al., 2012). Hydrophobicity is often measured using bacterial adherence to hydrocarbons (OTERO; OCAÑA; ELENA NADER-MACÍAS, 2004), with higher hydrophobicity typically correlated with increased adhesion capacity (THAKUR; ROKANA; PANWAR, 2016).

The potential for bacterial cell self-aggregation is another key feature of epithelial adhesion, as it enables nonspecific interactions—a prerequisite for mucosal colonization. Self-aggregation can be assessed using different methods, such cell line models like Caco-2 (RODRÍGUEZ-SÁNCHEZ et al., 2021). Enhanced adhesion to intestinal cells extends the colonization time, thereby increasing the likelihood of beneficial effects (THAKUR; ROKANA; PANWAR, 2016).

3.3 Production of beneficial and antimicrobial substances

Probiotics should produce beneficial substances such as organic acids, bacteriocins, and exopolysaccharides to inhibit pathogens and promote host health (COELHO; MALCATA; SILVA, 2022). As discussed earlier, antimicrobial activity is a significant functional criterion for competitively inhibiting pathogens (THAKUR; ROKANA; PANWAR, 2016).

Among the antimicrobial compounds produced by probiotic bacteria, organic acids—especially lactic and acetic acids—are the most prominent and well-characterized (SHOKRYAZDAN et al., 2017). LAB, for example, promote beneficial shifts in the gut microbiome by promoting the colonization of beneficial microorganisms and reducing pathogenic populations. This effect is attributed primarily to the production of organic acids, which lower the pH of the gastrointestinal tract (AROUTCHEVA et al., 2001).

Another key group of antimicrobial compounds produced by LAB are bacteriocins—small ribosomally synthesized antimicrobial peptides produced by specific bacterial strains.

Bacteriocins exhibit antimicrobial activity against other bacteria, while producer cells are immune to their own bacteriocins (DABA; ELKHATEEB, 2024).

4. Demonstration of benefit

4.1 Clinical studies

The efficacy of a probiotic strain must be validated through controlled and randomized clinical trials that demonstrate clear health benefits, such as improved digestion, strengthened immune response, or relief from intestinal disorders (SNIFFEN et al., 2018). The beneficial effect of a probiotic must be explicitly linked to the demonstration of a functional or health-promoting property. A functional property claim refers to the metabolic or physiological role that a nutrient or non-nutrient plays in the growth, development, maintenance, or other normal functions of the human or animal body (ANVISA, 2021).

To confirm the efficacy of a probiotic bacterial strain, it is essential to establish causality between probiotic consumption and the claimed effect. Studies conducted on humans are critical when probiotics are intended for human consumption (ANVISA, 2021). Determining the mechanism of action is another significant aspect, including processes such as immunomodulation, inhibition of pathogen colonization, stabilization of intestinal barrier function, and microbiota modulation (BINDA et al., 2020).

5. Final considerations

The screening of bacterial strains with probiotic potential involves complex processes that require a thorough evaluation of several criteria. Key factors include immune system modulation and pathogen antagonism, safety, survival in the gastrointestinal tract, adhesion to intestinal epithelial cells, and functional efficacy.

The safety of probiotic strains is crucial, necessitating the absence of transmissible antibiotic resistance genes and toxins. The ability to withstand the harsh conditions of the GTI, including stomach acidity and digestive enzymes, is essential to ensure that bacteria reach the intestine in sufficient quantities to exert their beneficial effects. Adhesion to intestinal epithelial cells is a critical factor as it facilitates colonization and direct interaction with the host. Furthermore, probiotic strains should demonstrate proven functional efficacy through *in vitro, in vivo,* and clinical studies. Desirable characteristics include the ability to inhibit pathogens, stimulate the immune system, and contribute to overall health maintenance.

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CHAPTER - 2: Searching for antibiotic-susceptible bioprotective lactic acid bacteria to control dangerous biological agents in Artisanal Cheese

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Abbreviations:

MAC= Minas Artisanal Cheese

BAC= Brazilian Artisanal Cheeses

LAB= Lactic Acid Bacteria

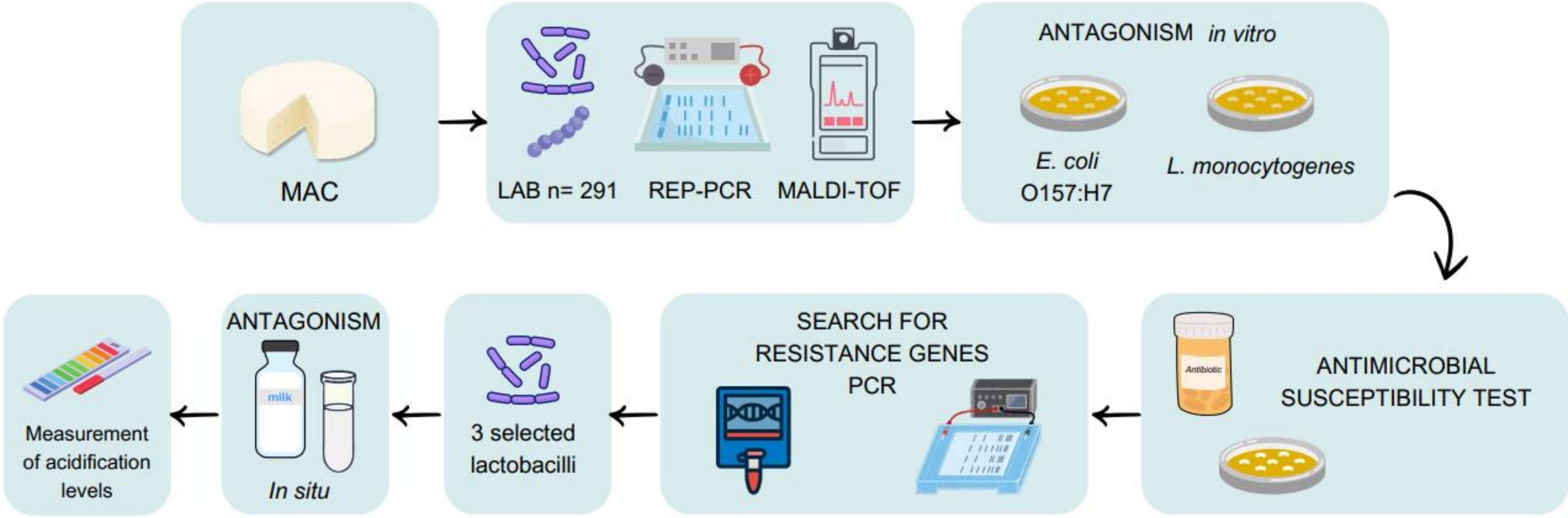
MAPA= Ministry of Agriculture, Livestock and Supply

ABSTRACT:

Minas artisanal cheese (MAC) samples (n=59) from 16 municipalities across five traditional MAC-producing regions in Brazil were used to prospect antibiotic-susceptible protective lactic acid bacteria (LAB) against three pathogenic bacteria found in the MAC. From 291 LAB isolates, 84 genetically diverse strains were selected via rep-PCR. MALDI-TOF identification revealed multiple species, predominantly *Enterococcus faecalis* (n = 37), *Enterococcus faecium* (n = 21), *Lactiplantibacillus plantarum* (*n* = 5) and *Lacticaseibacillus rhamnosus* (*n* = 3). The antagonistic activity of these strains was evaluated against *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 5779, and *Escherichia coli* O157:H7 using spot-on-lawn assays. Several strains showed strong inhibitory effects against *E. coli* and *L. monocytogenes*, with halo/colony ratios reaching 4.86 and 4.47, respectively. No antimicrobial peptide producing strain was observed. Antibiotic susceptibility was tested against nine antibiotics, and five strains were susceptible to all antibiotics, while 53 strains were susceptible to 5-8 antibiotics. However, five strains were resistant to all antibiotics, showing the highest resistance to gentamicin (66.7%), cotrimoxazole (58.3%), and streptomycin (57.2%). Resistance genes (*aacA-aphD*, *ermA/B*, *tetM/O/K/L/S*, *blaZ*, and *vanA/B*) were screened, and 40 strains harbored at least one gene. Taken together, these results revealed three antibiotic-susceptible bioprotective lactobacilli (strains 52, 177 and 272G) as superior strains whose effectiveness in eliminating *E. coli* O157 and *Listeria monocytogenes* in the milk matrix for up to 7 days was confirmed. These findings confirm the potential of these autochthonous lactobacilli to improve the safety of dairy, paving the way for their applications in product development in future projects.

Keywords: antimicrobial activity, antibiotic resistance, lactobacilli, enterococci, *Listeria monocytogenes*, *Escherichia coli* O157:H7.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Bioprotective microorganisms play an important role in food safety and quality due to their ability to inhibit the growth of undesirable microorganisms through the production of antimicrobial metabolites, including organic acids, hydrogen peroxide, acetoin, diacetyl, reuterin, and bacteriocins (Bintsis & Papademas, 2024; Souza et al., 2022). These microorganisms have gained global attention for their application in food preservation, extending shelf-life, and ensuring food safety (Ben Said et al., 2019; Hosken et al., 2023). As a sustainable alternative to chemical preservatives, biopreservation aligns with the increasing consumer demand for clean-label products (Bintsis & Papademas, 2024). The use of bioprotective strains in food production requires not only the demonstration of their functional efficacy but also confirmation of their safety through rigorous scientific studies, which must include testing for antibiotic susceptibility and the absence of pathogenic genes and genes encoding resistances to clinically-relevant antibiotics (Fischer & Titgemeyer, 2023).

Among bioprotective microorganisms, lactic acid bacteria (LAB) are particularly notable for their extensive application in food production, especially in fermented dairy products (Zarzecka et al., 2022). Various genera and species of LAB, including Enterococcus, Lactobacillus, Lactococcus, Pediococcus, Streptococcus, Weissella, and Leuconostoc, are commonly found in milk and cheese. Within this group, Lactobacillus species are of particular importance due to their frequent isolation from fermented dairy products and their well-documented history of safe use, being generally recognized as safe (GRAS) (Margalho et al., 2021; Samedi & Linton Charles, 2019). In contrast, Enterococcus spp. represent the most contentious members of the LAB group. While certain strains have been utilized as bioprotective agents and play a crucial biotechnological role in cheese production (Aspri et al., 2017; Pingitore et al., 2012), others are associated with pathogenic potential (O'Driscoll & Crank, 2015). This duality stems from the possible presence of genes encoding toxins, virulence factors, and antimicrobial resistance (Krawczyk et al., 2021). Consequently, rigorous screening of Enterococcus strains for both phenotypic and genotypic traits is essential to ensure their safety in food applications.

Cheese, particularly artisanal varieties, provides a favorable medium for LAB proliferation and activity due to its high nutritional content and specific environmental conditions. However, these same characteristics—such as high-water content, water activity, pH values ranging from 5.0 to 5.4, and extensive handling—significantly increase the risk of contamination, even in cheeses made from pasteurized milk (Antônio & Borelli, 2020;

Sandoval & Ribeiro, 2021). The use of raw milk in artisanal cheese production further amplifies these safety challenges. Pathogens such as Shiga-toxin producing Escherichia coli, coagulase-positive staphylococci, Salmonella spp., and Listeria monocytogenes have been detected in Brazilian artisanal cheeses (Allaion et al., 2021; Carvalho et al., 2014; Firmo et al., 2023). These concerning findings highlight the pressing need for additional control measures, such as the application of bioprotective cultures, to enhance the safety and quality of cheese production.

Minas artisanal cheese (MAC) is a traditional product from Minas Gerais, the largest artisanal cheese-making state in Brazil, which has significant socioeconomic importance for the region once the activity comprises a source of income for rural families (Kamimura et al., 2019). MAC is produced using fresh, raw cow milk and natural lactic acid cultures, adhering to strict standards that emphasize its firm consistency, characteristic flavor, and absence of artificial additives according to the historical and cultural tradition of the region of origin (Agricultural Institute of Minas Gerais [IMA], 2024). Factors such as climate, livestock feeding practices, and animal genetics influence MAC characteristics, contributing to its unique microbiota and sensory characteristics (Campos et al., 2023).

The endogenous microbiota of MAC, particularly LAB, plays a pivotal role in defining its identity (Nero et al., 2021). Ten regions in Minas Gerais are officially recognized as MAC producers (IMA, 2024), with Araxá, Campo das Vertentes, Cerrado, Serra da Canastra, Serra do Salitre, Serro, and Triângulo Mineiro standing out due to their distinctive geomorphological and cultural characteristics, collectively referred to as terroir, which significantly influence LAB composition (Nero et al., 2021; Pineda et al., 2021). Interest in isolating autochthonous LAB from MAC has grown, aiming to harness their bioprotective properties in cheese production (Hosken et al., 2023).

Although Brazil is recognized as a megadiverse country and one of the largest producers of milk and dairy products globally, it remains heavily reliant on imported bioinputs for its dairy industry (Campos et al., 2023). This scenario underscores the urgent need for investments in research, development, and innovation (RD&I) to explore the country's diverse LAB strains. This study addresses this gap by identifying antibiotic-susceptible bioprotective LAB strains isolated from MAC to control the hazardous pathogens and contaminants—Listeria monocytogenes, Escherichia coli O157:H7, and Enterococcus faecalis—in artisanal cheese production.

2. MATERIAL AND METHODS

2.1 Isolation of Lactic Acid Bacteria

MAC (*n* =59) from 16 municipalities of the Minas Gerais traditional microregions, Canastra, Serro, Araxá, Triângulo and Cerrado, were analyzed. Before plating, each cheese sample was diluted in 2% sodium citrate solution (Synth, Brazil), homogenized and then diluted in phosphate buffer solution (Merck, Germany). LAB prospecting was performed on MRS agar (BD Difco, USA) and M17 agar (Sigma–Aldrich, USA) at 35 °C for 24 - 48 hours. Bacterial colonies considered phenotypically distinct were picked from agar plates and purified on MRS or M17 media, according to the culture medium of origin, at 35 °C for 24 h. The isolates were then tested for catalase production, subjected to Gram staining and submitted to microscopic examination. All catalase-negative and Gram-positive cocci or bacilli were presumably considered LAB. Finally, the bacteria were transferred to microtubes containing Litmus Milk (Sigma–Aldrich, USA) and stored at -20 °C and -80 °C until further characterization.

2.2 rep-PCR fingerprinting

To evaluate genetic similarity and exclude replicates, LAB isolates were grouped by repetitive extragenic palindromic PCR (rep-PCR) according to Versalovic et al. (1994). Rep-PCR was performed with the universal (GTG)5 primer (5'-GTG GTG GTG GTG GTG-3'). Banding patterns were examined with the Bionumerics software program (version 6.6, Applied Maths, Sint-Martens-Latem, Belgium) using Jaccard's coefficient. Cluster analyses of data obtained via rep-PCR were achieved using the unweighted pair group method with arithmetic averages (Leite et al., 2015).

2.3 Identification by MALDI-TOF

Taxonomic identification of genetically distinct LAB strains was carried out, via matrix-assisted laser desorption ionization – time of flight (MALDI-TOF), following the procedure described by Nacef et al. (2024). MS profiles were acquired in triplicate from each bacterial culture. Two hundred microliters of bacterial suspension were collected and washed twice with sterile Milli-Q water (16,000 g × 1 min). The supernatant was discarded, and the cell pellet was gently resuspended in 300 µL of sterile Milli-Q water. Absolute ethanol (900 µL) (Sigma–Aldrich) was added for bacterial inactivation. The cell suspension was centrifuged (16,000 g × 2 min), the supernatant was discarded, and the cell pellet was dried (room temperature/10 min). Cellular proteins were extracted by adding 10 µL of 70% formic acid (Sigma–Aldrich) to the sediment, followed by the addition of 10 µL of pure acetonitrile (Sigma–Aldrich). After mixture homogenization, another centrifugation (16,000 g × 2 min) was performed and 1 µL of the supernatant was applied to a well of the MALDI plate (Bruker Daltonics) and dried at room temperature. Each sample was coated with 1 µL of the matrix for MALDI (10 mg/mL of α-19 cyano-4-hydroxycinnamic acid - Sigma–Aldrich), in a solution containing 50% acetonitrile and 2.5% trifluoroacetic acid (v/v)), followed by air drying. Mass spectra were acquired via an Autoflex III SmartBeam mass spectrometer (Bruker Daltonics) equipped with a 200 Hz laser, operating in positive linear mode, with a mass range of 2,000-20,000 Daltons. External calibration was performed using Bacterial Test Standard calibrant mix (Bruker Daltonics). The equipment parameters included an IS1 source voltage of 20 kV, an IS2 source voltage of 18.55 kV, a lens voltage of 8.80 kV, and an ion extraction delay time of 240 ns. Random laser shots were performed with a peak sampling rate of 0.5GS/s, totaling 2000 spectra, which were summed and processed via the centroid peak detection algorithm of the FlexControl 3.3 program (Bruker Daltonics). MS spectra identification, using the Bruker database, resulting in the raw spectrum of each sample, which was considered high confidence when the score values were between 3.0 and 2.0; medium confidence when the scores were between 1.70 and 1.99; and scores lower than 1.69 as nonidentifiable. As a positive control, *Escherichia coli* ATCC 8739 was used.

2.4 Assessment of the antagonistic activity of LAB strains against pathogens

The antagonistic activity of the nonredundant LAB strains was determined via spot-on-lawn method as proposed by Tagg et al. (1976) with modifications. Three indicator pathogens were used: *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848 and *Listeria monocytogenes* ATCC 5779.

LAB strains were subsequently grown on MRS (35 °C/24 h). After growth, three colonies of each LAB were transferred to 1,000 µL of MRS broth and incubated at 35 °C ± 2 °C for 24 h. Finally, 5 µL of the LAB suspension were added to MRS agar at five equidistant spots. The plate was incubated aerobically at 35 ± 2 °C for 24 hours. The indicator bacteria were inoculated into 10 mL of BHI broth and incubated at 37 ºC for 4 hours. Then, 100 µL of the pathogen culture was added to 7 mL of 50 °C prewarmed semisolid BHI agar, and the mixture was poured onto the MRS plate, forming a layer over the spots. The Petri dishes were reincubated aerobically for 14 to 18 hours at 35 °C ± 2 °C. The presence of halo around the spots was considered favorable evidence of antibacterial activity against the indicator (Lewus & Montville, 1991). Halos were measured in millimeters (mm). Strains exhibiting a halo/colony ratio of less than 1 were classified as nonantagonistic (-). Strains with a halo/colony ratio greater than 1 were considered to display regular antagonistic activity (+). Those with a halo/colony ratio exceeding 2 were deemed to have good antagonistic activity (++), and strains with a halo/colony ratio greater than 3 were categorized as having excellent antagonistic activity (+++).

2.5 Assessment of the chemical nature of the inhibitory substances

Antimicrobial susceptibility tests were conducted via the disc diffusion method as originally described by Bauer et al. (1966), with modifications as outlined by Santos et al. (2015). LAB strains were reactivated in MRS agar for 24 h at 37 ºC to obtain isolated colonies. After growth, 3 colonies were collected, inoculated on MRS broth and incubated for 24 h at 37 ºC. After growth, suspensions of all strains were diluted to 0.5 (108 CFU/mL) on the McFarland scale in a saline solution, and 100 μL of each culture was inoculated into Petri dishes containing 25 mL of MRS agar and spread with a Drigalski loop throughout the culture medium surface on which 9 discs containing antimicrobials (Oxoid®, Basingstoke, England) were placed. The antimicrobials tested were penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), and erythromycin (15 μg), in addition to co-trimoxazole (1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole). These antibiotics were selected based on their clinical relevance and use in the treatment of diseases, such as bovine mastitis, within dairy production systems, in accordance with guidelines established by regulatory agencies and evidence from previous scientific studies. The plates were incubated aerobically for 24 h at 35 ºC. To evaluate the results, inhibition zones (mm) were measured using a millimeter ruler. Based on these measurements, the strains were classified as either sensitive or resistant to each antimicrobial agent, following the criteria established by Charteris et al. (1998) and Nalepa and Markiewicz (2023). This classification was necessary because no specific guidelines exist for lactic acid bacteria under CLSI or EUCAST (Nalepa & Markiewicz, 2023).

2.6 Assessment of the susceptibility of LAB strains to antimicrobials

Antimicrobial susceptibility tests were conducted via the disc diffusion method as originally described by Bauer et al. (1966), with modifications as outlined by Santos et al. (2020). LAB strains were grown in MRS agar for 24 h at 37 ºC to obtain isolated colonies. After growth, 3 colonies were collected, inoculated on MRS broth and incubated for 24 h at 37 ºC. After growth, 100 μL of each culture was inoculated into Petri dishes containing 25 mL of MRS agar and spread with a Drigalski loop throughout the culture medium surface on which 9 discs containing antimicrobials (Oxoid®, Basingstoke, England) were placed. The antimicrobials tested were penicillin G (10 µg), ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), and erythromycin (15 µg), in addition to co-trimoxazole (1.25 µg of trimethoprim and 23.75 µg of sulfamethoxazole). The plates were incubated aerobically for 24 h at 35 ºC. To evaluate the results, inhibition zones (mm) were measured using a millimeter ruler. On the basis of these measurements, strains were classified as either sensitive or resistant to each antimicrobial agent, following the criteria established by Charteris et al. (1998).

2.7 Detection of antibiotic resistance genes in LAB strains

The detection of antibiotic resistance genes (*erm*A/B, *tet*O/M/K/L/W/S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A/B) was performed via PCR according to protocols described in the literature (Table 1). Colonies were collected using disposable plastic tips (with a volume of 200 µL) and then introduced directly into the PCR mixture.

Table 1. Primers used to detect antibiotic resistance genes in LAB strains.

Target gene	Primer (5'-3')	Amplicon size (bp)	Reference
*erm*A	*erm*A 1 - GTTCAAGAAC AATCAATACAGAG *erm*A 2 - GGATCAGGAA AAGGACATTTTAC	421	Lina et al., 1999
*erm*B	*erm*B 1 - CGTTTACGAAATTGGAACAGGTAAAGGGC *erm*B 2 - GAATCGAGACTTGAGTGTGC	359	Lina et al., 1999
*tet*O	*tet*O 1 - AACTTAGGCATTCTGGCTCAC *tet*O 2 - TCCCACTGTTCCATATCGTCA	515	Ng et al., 2001
*tet*M	*tet*M 1 - AGTTTTAGCTCATGTTGATG *tet*M 2 - TCCGACTATTTAGACGACGG	1862	Trzcinski et al., 2000
*tet*K	*tet*K 1 - TATTTTGGCTTTGTATTCTTTCAT *tet*K 2 - GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al., 2000
*tet*L	*tet*L 1 - ATAAATTGTTTCGGGTCGGTAAT *tet*L 2 - AACCAGCCAACTAATGACAATGAT	1077	Trzcinski et al., 2000
*tet*W	*tet*W 1 - GAGAGCCTGCTATATGCCAGC *tet*W 2 - GGGCGTATCCACAATGTTAAC	168	Aminov et al., 2001
*tet*S	*tet*S 1 - TGGAACGCCAGAGAGGTATT *tet*S 2 - ACATAGACAAGCCGTTGACC	740	Obioha et al., 2023
*aac*A-*aph*D	*aac*A-*aph*D 1 - TAATCCAAGAGCAATAAGGGC *aac*A-*aph*D 2 - GCCACACTATCATAACCACTA	228	Strommenger et al., 2003
*mec*A	*mec*A 1 - TGGCTATCGTGTCACAATCG *mec*A 2 - CTGGAACTTGTTGAGCAGAG	310	Vannuffel et al., 1995
*bla*Z	*bla*Z 1 - AAGAGATTTGCCTATGCTTC *bla*Z 2 - GCTTGACCACTTTTATCAGC	517	Vesterholm-Nielsen et al., 1999
*van*A	*van*A 1 - TCTGCAATAGAGATAGCCGC *van*A 2 - GGAGTAGCTATCCCAGCATT	377	Lemcke & Bülte, 2000
*van*B	*van*B 1 - GCTCCGCAGCCTGCATGGACA *van*B 2 - ACGATGCCGCCATCCTCCTGC	529	Lemcke et al., 2000

2.8 Selection criteria for superior LAB strains

LAB strains demonstrating excellent antagonistic activity against indicator pathogens, susceptibility to at least 6 of the 9 antibiotics tested, and the absence of any antibiotic resistance genes (*erm*A/B, *tet*O/M/K/L/W/S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A/B) were selected for further characterization. These selected strains were subsequently evaluated for their acidifying capacity and tested for antimicrobial activity against pathogens in the milk matrix.

2.9 Antagonistic activity in milk

To verify the antagonistic activity in food, selected LAB, as described in section 2.8, and the respective indicator pathogens were cocultured in sterile skim milk at 30 °C for up to 21 days. For this purpose, three isolated colonies of each LAB and each indicator bacteria were inoculated into 6 mL of MRS broth or BHI broth, respectively, and incubated at 35 °C for 6 hours. The cultures were then centrifuged at 10,000 × *g* 5 minutes, washed, and resuspended in 0.85% saline solution to obtain a suspension with a concentration of 10^8 colony-forming units (CFU)/mL. Subsequently, these suspensions were inoculated into 10 mL of milk in order to achieve final concentrations of 10^6 CFU/mL of each LAB and 10^4 or 10^6 CFU/mL of each pathogen. The following groups were established as controls: pure milk as a negative control, milk with each selected LAB strain, and milk with each indicator pathogen as positive controls. Cocultivation was performed by mixing combinations of each selected LAB with the respective indicator pathogen in the milk. To confirm the initial concentration of cells inoculated in milk, 0.5 mL of the samples were collected at time zero and serially diluted. Then, aliquots of 10 µL from at least four dilutions of each treatment and control were inoculated in triplicate on selective media specific for each species: MRS agar with $CaCO_3$ for LAB, MacConkey agar for *E. coli*, and ALOA agar for *L. monocytogenes*. The plates were incubated at 35 °C for 48 hours, in anaerobic jars for LAB only, and colony counts were performed to determine the number of CFU in each milk sample. To evaluate the inhibitory effect of LAB on pathogens, 0.5 mL aliquots from each tube containing milk (controls and treatments) were collected, diluted, and plated as described above at 24, 48, and 72 hours and at 7, 9, 14, and 21 days. The pH variation was also monitored during the same incubation period and at the same time intervals.

2.10 Statistical analysis

The experiments were conducted twice, with triplicate samples per test, and the results were as mean values. The antagonistic activity of the selected strains (52, 177, and 272G) against the tested pathogens, *E. coli* and *L. monocytogenes*, was evaluated using ANOVA to assess overall significant differences. Tukey test was applied with a significance level of 5% ($p < 0.05$). All analyses were performed using RStudio software (RStudio Inc., Boston, MA, USA).

3. RESULTS

3.1 Isolation, genetic diversity and identification of LAB

A total of 291 Gram-positive, catalase-negative bacterial colonies were isolated from 59 cheese samples (Fig.1) and presumably designated as LAB isolates. From this collection, 84 genetically nonredundant strains were selected on the basis of genetic dissimilarity assessed by rep-PCR (Fig. 2) to mitigate clonality and establish the bacterial collection used in this study. The identification of genetically nonredundant strains at the species level, according to the cheese sample origin, is summarized in Table 2. The MALDI-TOF identification scores ranged from 1.7 to 2.38, indicating medium to high confidence levels. The majority of the isolated species were *Enterococcus* spp. (*n* = 65), followed by the lactobacilli group (*n* = 10), *Streptococcus* sp. (*n* = 2), *Lactococcus* sp. (*n* = 1), *Leuconostoc* sp. (*n* = 1), and *Pediococcus* sp. (*n* = 1).

Data regarding the origin of the MAC samples and the LAB isolation results by region are presented in Fig. 1. The majority of strains were obtained from Serro (*n* = 59), whereas only two LAB were isolated from the Cerrado. The primary species or groups observed were as follows: Araxá: *Enterococcus faecium* (63.6%); Canastra: *Enterococcus* spp. (83.3%); Cerrado: *Streptococcus infantarius* (50%) and *Enterococcus faecalis* (50%); Serro: 77.2% enterococci and 14.0% lactobacilli; and Triângulo: 66.3% *Enterococcus* spp. and 33.3% *Lactococcus lactis*. The distribution of LAB strains for each MAC-producing region was as follows: *E. faecium (n* = 7*), E. faecalis (n* = 2*)* and *Lacticaseibacillus rhamnosus (n* = 1*)* from Araxá; *E. faecalis (n* = 2*), E. faecium (n* = 1*), Enterococcus casseliflavus (n* = 1*), Enterococcus sulfureus* (*n* = 1) and *Lactiplantibacillus plantarum (n* = 1*)* from Canastra; *E. faecalis (n* = 1*)* and *S. infantarius* (*n* = 1) from Cerrado; *E. faecalis (n* = 27*), E. faecium (n* = 13*), Enterococcus hirae (n* = 2*), Enterococcus durans (n* = 1*), Enterococcus mundtii (n* = 1*), L. plantarum (n* = 4*), L. rhamnosus (n* = 2*), Lactilactobacillus curvatus (n* = 2*), Pediococcus pentosaceus (n* = 1*)* and *Streptococcus gallolyticus (n* = 1*)* from Serro; and *E. faecalis (n* = 1*), E. casseliflavus (n* = 1*),* and *L. lactis (n* = 1*)* from Triângulo.

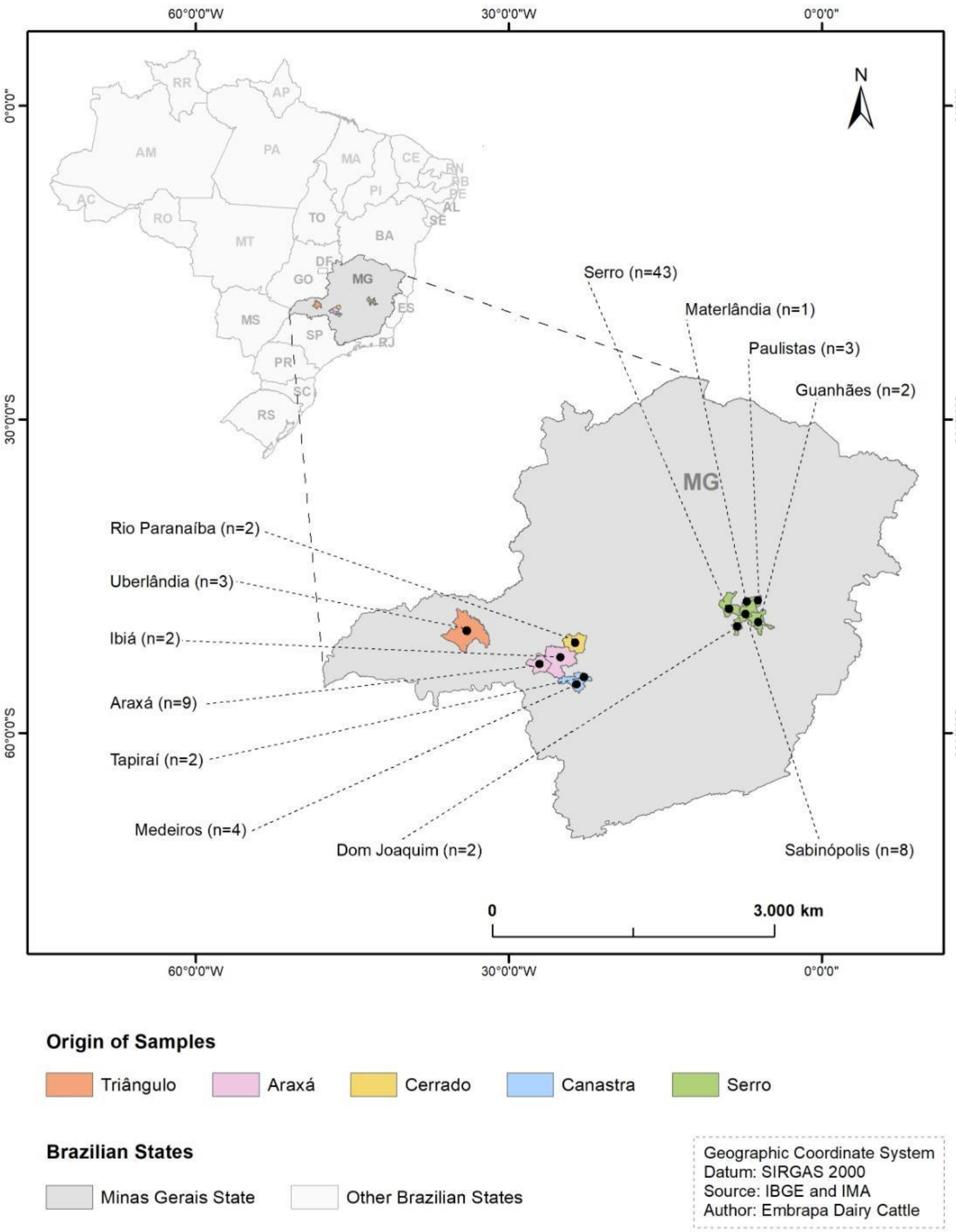


Fig 1. Map representing the origin of minas artisanal cheeses (MAC) samples and the results of the LAB isolates for each municipality from the regions studied.

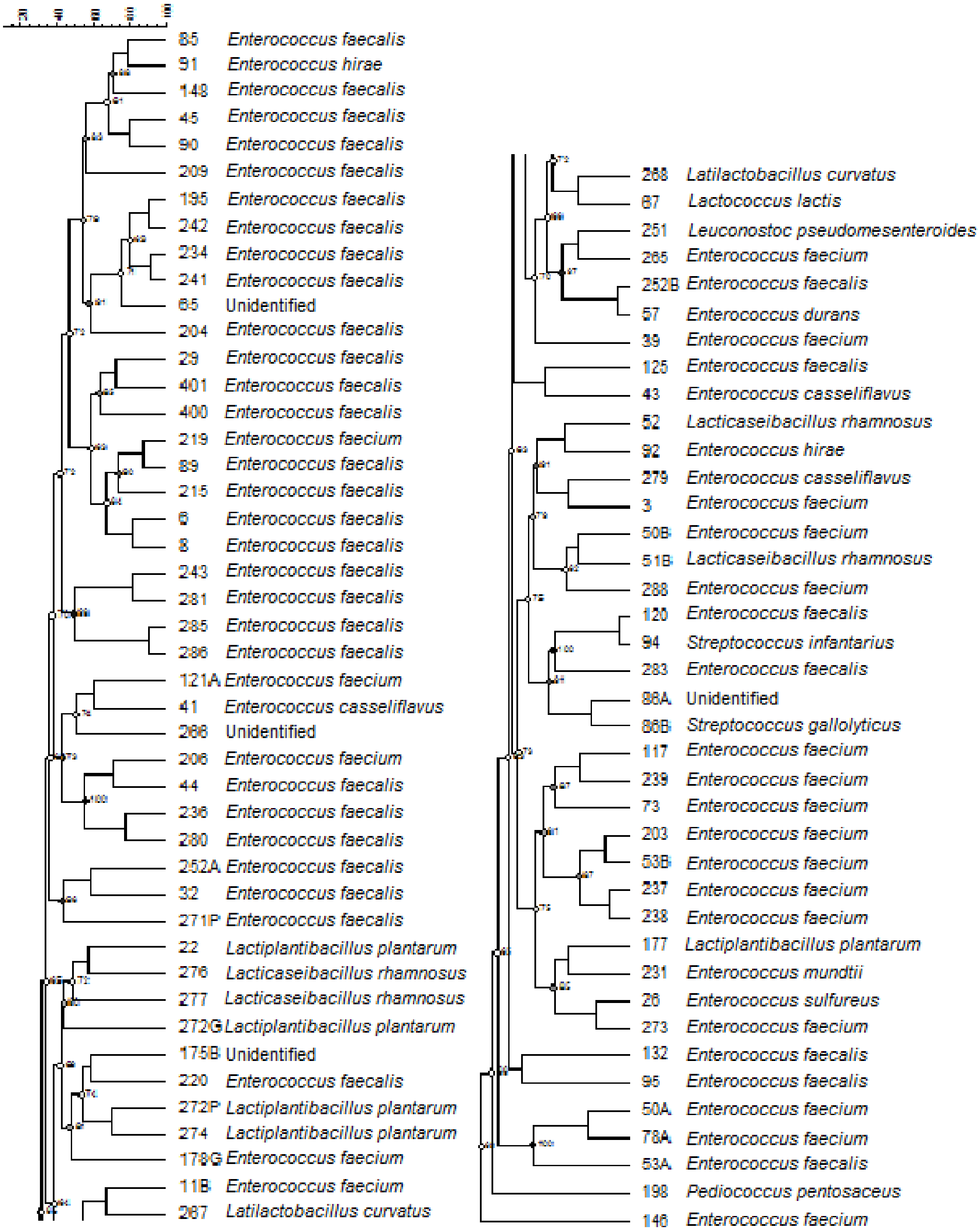


Fig 2. Dendrogram based on the cluster analysis of rep–PCR fingerprints from lactic acid bacteria (LAB) used in the study. The image was generated by the Bionumerics software program (version 6.6, Applied Maths, Sint-Martens-Latem, Belgium).

3.2 Antagonistic activity of LAB strains against indicator pathogens

The antagonistic capacity of the genetically nonredundant LAB strains, verified via the spot-on-lawn technique, is presented in Table 2 and Figure 3. The strains exhibiting the best antagonistic activities against *E. coli* O157:H7 IAL1848 and *L. monocytogenes* ATCC 5779 belong to the lactobacilli group, with halo/colony ratios exceeding 4, which is regarded as having excellent antagonistic capacity according to the criteria established in this study. *E. faecalis* ATCC 29212 was the most resistant pathogen, showing no susceptibility to 14.3% of the LAB strains tested. All LAB strains showed at least regular antagonistic activity (halo/colony ratio > 2) against *L. monocytogenes,* and 69 of them showed antagonistic activity against all indicator pathogens tested.

In the enterococci group (*n* = 65), the majority (87.7%) exhibited antagonistic activity ranging from regular to excellent against *E. faecalis* ATCC 29212, *E. coli* O157:H7 IAL1848 and *L. monocytogenes* ATCC 5779. Only *E. faecalis* (strains 8, 220 and 252B), *E. faecium* (strains 11B, 39 and 117 and 265), and *E. sulfureus* (strain 26) did not antagonize the indicator pathogens *E. faecalis* ATCC 29212 and *E. coli* O157:H7 IAL1848, respectively. Among the *Lactobacillus* strains *(n* = 10), *L. plantarum* and *L. rhamnosus* showed the best protective potential, exhibiting excellent antagonistic activity against all the indicators tested, with some isolates achieving a halo/colony ratio greater than 4. Conversely, although *L. curvatus* demonstrated regular and good antagonistic activity against *E. coli* O157:H7 IAL1848 and *L. monocytogenes* ATCC 5779, respectively, this species did not show antagonism against the indicator pathogen *E. faecalis* ATCC 29212.

Among the other isolated species, *Pediococcus pentosaceus* exhibited excellent antagonism against *E. coli* O157:H7 IAL1848 and *L. monocytogenes* ATCC 5779. Additionally, *L. lactis* demonstrated a halo/colony ratio greater than 3 for *L. monocytogenes* ATCC 5779, and *E. mundtii* presented a halo/colony ratio exceeding 4 against *E. coli* O157:H7 IAL1848. With respect to individual antagonistic activity, certain lactobacilli strains displayed high inhibitory capacity against indicator pathogens. Notably, *L. plantarum* (strains 17, 272G and 274) and *L. rhamnosus* (strain 52) achieved halo/colony ratios greater than 4 against both *E. coli* O157:H7 IAL1848 and *L. monocytogenes* ATCC 5779.

Concerning the chemical nature of the inhibitory substances, no retraction or absence of inhibition halos was observed at the application points of the proteinase K, pepsin and amylase enzymes in any of the 84 strains studied. These results suggest that the compounds responsible for the antagonistic activity likely do not have a proteinaceous nature, based on the proteases used.

Table 2. Identification by MALDI-TOF, microregion of isolation, and antagonistic activity (halo/colony ratio) of the lactic acid bacteria (LAB) strains against the indicator pathogens *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848 and *Listeria monocytogenes* ATCC 5779.

MALDI-TOF identification LAB species tested	n[1]	Region (s) of MAC sample (s) origin	Indicator pathogen Halo/colony average followed by the range of values[2*]		
			Enterococcus faecalis	*Escherichia coli*	*Listeria monocytogenes*
Enterococcus faecalis	37	Araxá, Canastra, Cerrado, Serro, Triângulo	1.65 [0.0 - 3.0] (+)	2.59 [1.47 - 3.75] (++)	2.84 [2.03 - 3.7] (++)
Enterococcus faecium	21	Araxá, Canastra, Serro	1.47 [0.0 - 2.7] (+)	2.20 [0.0 - 3.76] (++)	2.68 [1.07 - 3.46] (++)
Enterococcus hirae	2	Serro	2.02 [1.88 - 2.17] (++)	1.87 [1.73 - 2] (+)	2.9 [2.83 - 2.95] (++)
Enterococcus durans	1	Serro	1.38 (+)	2.25 (++)	2.8 (++)
Enterococcus casseliflavus	2	Canastra, Triângulo	1.32 [1.21 - 1.43] (+)	2.16 [1.95 - 2.36] (++)	3.09 [2.37 - 3.8] (+++)
Enterococcus mundtii	1	Serro	1.63 (+)	4.05 (+++)	3.0 (+++)
Enterococcus sulfureus	1	Canastra	1.83 (+)	0.0 (-)	2.46 (++)
Lactiplantibacillus plantarum	5	Canastra, Serro	2.48 [2.13 - 2.71] (++)	4.3 [3.56 - 4.86] (+++)	4.1 [4.0 - 4.47] (+++)
Lacticaseibacillus rhamnosus	3	Araxá, Serro	2.26 [1.75 - 2.75] (++)	3.30 [2.26 - 4.6] (+++)	3.43 [3.03 - 4.0] (+++)
Lactilactobacillus curvatus	2	Serro	0.0 (-)	1.59 [0.0 - 3.18] (+)	2.31 [1.72- 2.89] (++)
Lactococcus lactis	1	Triângulo	0.0 (-)	1.79 (+)	3.15 (++)
Leuconostoc pseudomesenteroides	1	Serro	0.0 (-)	0.0 (-)	2.0 (++)
Pediococcus pentosaceus	1	Serro	1.9 (+)	3.12 (+++)	3.12 (+++)
Streptococcus gallolyticus	1	Serro	1.25 (+)	2.09 (++)	2.36 (++)
Streptococcus infantarius	1	Cerrado	0.00 (-)	2.40 (++)	2.34 (++)
Not identified	4	Araxá, Serro	1.12 [0.0 - 1.78] (+)	1.57 [0.0 - 2.45] (+)	2.70 [2.4 - 3.05] (++)
Total of strains	84	Araxá, Canastra, Cerrado, Serro, Triângulo	1.57 [0.0 - 3.0] (+)	2.48 [0.0 - 4.86] (++)	2.90 [1.0 - 4.5] (++)

LAB n[1]: Number of LAB strains isolated by species.
Halo/colony ratio[2]: Halo/colony ratio average per LAB species, followed by the lowest and highest values in the range (in parentheses).
*Halo/colony ratio < 1 = nonantagonistic (-); Halo/colony ratio > 1 = regular antagonistic activity (+); > 2 = good antagonistic activity (++); and > 3 = excellent antagonistic activity (+++).

Fig 3. Antagonistic activity of LAB strains against *Escherichia coli* O157:H7 IAL1848. The halo (dashed orange arrow)/colony (solid green arrow), represented in **Fig. 3A**, shows the antagonistic activity of the strain *Lactiplantibacillus plantarum* strain 177. The red arrow in **Fig. 3B** highlights the LAB strain 65, which did not show antagonistic activity against *E. coli*.

3.3 LAB strains susceptibility to antimicrobials and resistance gene detection

The antimicrobial resistance data, antibiotic susceptibility profiles and detected resistance genes are detailed in Table 3. Most of the LAB strains were susceptible to chloramphenicol (79.8%), ampicillin (78.1%), and erythromycin (72.6.3%). In contrast, the majority of LAB isolates were resistant to gentamicin (66.70%), cotrimoxazole (58.3%) and streptomycin (57.2%). Susceptibility profiles are expressed in terms of LAB species and their susceptibility to 0 to 9 of the nine antibiotics tested (profiles X to I, respectively). Most strains (55.9%) were susceptible to 6 out of the 9 antibiotics tested (profiles IV, III, II, and I, respectively). The enterococci strains *E. faecium* (strains 73 and 117), *E. faecalis* (strains 90 and 195) and *E. sulfureus* (strain 26) were susceptible to all the antibiotics tested. Only five strains were resistant to all the antibiotics tested: *E. faecium* (strain 206), *E. faecalis* (strain 252A), *L. curvatus* (strain 268), *L. rhamnosus* (strain 276) and an unidentified strain (strain 175B). Enterococci and lactobacilli species demonstrated high diversity in terms of antibiotic susceptibility, presenting different profiles.

Among the genes detected, *aac*A-*aph*D was the most common (34.7%) gene in the strains tested, followed by the genes *tet*M (20%), *tet*S (14.3%) and *erm*A (10.7%). No strains carrying the *erm*B, *tet*O, *tet*K, *tet*L, *tet*W, *mec*A, *bla*Z, *van*A or *van*B genes were identified.

Table 3. Susceptibility of LAB species to antibiotics (ampicillin, penicillin, chloramphenicol, cotrimoxazole, erythromycin, streptomycin, gentamicin, tetracycline and vancomycin), determined via the disc diffusion method, and susceptibility profiles and resistance genes were detected.

LAB species	LAB *n*[1]	Number of LAB strains susceptible to the antibiotics tested[2]									Susceptibility Profile[3]	Resistance genes detected[4]
		AMP	PEN	CHL	SXT	ERI	STR	GEN	TET	VAN		
Enterococcus faecalis	37	33	26	27	14	27	11	13	22	34	I, II, III, IV, V, VI, VII, VIII, and X	*aac*A-*aph*D (7/35), *erm*A (1/16), *tet*M (9/33)
Enterococcus faecium	21	14	7	18	11	13	15	11	11	16	I, II, III, IV, V, VI, VIII, and X	*aac*A-*aph*D (12/20), *erm*A (2/7), *tet*M (6/20)
Enterococcus hirae	2	1	0	2	0	2	0	0	2	0	VI and VII	*aac*A-*aph*D (2/2)
Enterococcus durans	1	1	1	1	0	1	0	1	1	1	III	None
Enterococcus casseliflavus	2	1	1	2	0	1	0	0	1	2	IV and VIII	None
Enterococcus mundtii	1	0	0	0	0	0	0	0	1	0	IX	*aac*A-*aph*D (1/1)
Enterococcus sulfureus	1	1	1	1	1	1	1	1	1	1	I	None
Lactiplantibacillus plantarum	5	5	2	5	5	5	5	5	4	0	II, III and IV	*aac*A-*aph*D (1/4)
Lacticaseibacillus rhamnosus	3	2	0	2	2	2	2	2	1	1	III and X	*aac*A-*aph*D (1/3)
Lactilactobacillus curvatus	2	1	1	1	0	1	0	0	1	0	V and X	*aac*A-*aph*D (1/1)
Lactococcus lactis	1	1	1	1	0	1	1	1	0	1	III	*tet*S (1/1)
Leuconostoc pseudomesenteroides	1	1	1	1	0	1	0	0	1	0	V	None

Pediococcus pentosaceus	1	1	0	1	0	1	0	1	1	0	V	None
Streptococcus gallolyticus	1	0	0	1	0	1	0	0	1	1	IV	*aac*A-*aph*D (1/1)
Streptococcus infantarius	1	1	1	1	1	1	0	0	1	1	III	None
Not identified	4	2	2	3	1	3	1	3	2	2	III, V and X	*tet*S (1/2)
Total of strains	84	65	44	67	35	61	36	28	51	60	I, II, III, IV, V, VI, VII, VIII, IX and X	*aac*A-*aph*D: 34.7% *erm*A: 10.7% *tet*M: 20% *tet*S: 14.3%
Total of strains %	100	78.1	52.4	79.8	41.7	72.6	42.8	33.3	60.7	71.4		

[1]: Number of LAB strains isolated by species.

[2]: AMP = Ampicillin; PEN = Penicillin; CHL = Chloramphenicol; SXT = Cotrimoxazole; ERI = Erythromycin; STR = Streptomycin; GEN = Gentamicin; TET = Tetracycline; VAN = Vancomycin.

[3]: I = Susceptible to all antibiotics tested; II = susceptible to 8/9 antibiotics tested; III = susceptible to 7/9 antibiotics tested; IV = susceptible to 6/9 antibiotics tested; V = susceptible to 5/9 antibiotics tested; VI = susceptible to 4/9 antibiotics tested; VII = susceptible to 3/9 antibiotics tested; VIII = susceptible to 2/9 antibiotics tested; IX = susceptible to 1/9 antibiotics tested and X = susceptible to 0/9 antibiotics tested.

[4]: Strain number tested for each resistance gene: *aac*A-*aph*D (75), *erm*A (28), *erm*B (84), *tet*O (79), *tet*M (79), *tet*K (79), *tet*L (4), *tet*W (4), *tet*S (14), *mec*A (4), *bla*Z (79), *van*A (4) and *van*B (4); gene detection frequency.

Table 4. LAB strains selected, and their description, antagonistic activity (halo/colony ratio in mm) against the indicator pathogens *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848 and *Listeria monocytogenes* ATCC 5779, susceptibility profile, and resistance genes detected.

LAB strain		*Lacticaseibacillus rhamnosus* Code 52	*Lactiplantibacillus plantarum* Code 177	*Lactiplantibacillus plantarum* Code 272G	Mean	Standard deviation	***p***[3]
Microregion		Araxá	Serro	Serro			
Morphology		Long rod cell	Rod-shaped cell	Short rod cell			
Indicator pathogen	*E. coli*	4.60	4.73	4.68	4.67	0.066	0.015
	L. monocytogenes	4	4.47	4	4.16	0.270	0.029
Susceptibility Profile[1]:		III	IV	III			
Resistance genes detected[2]		None	None	None			

[1]: I = Susceptible to all antibiotics tested; II = susceptible to 8/9 antibiotics tested; III = susceptible to 7/9 antibiotics tested; IV = susceptible to 6/9 antibiotics tested; V = susceptible to 5/9 antibiotics tested; VI = susceptible to 4/9 antibiotics tested; VII = susceptible to 3/9 antibiotics tested; VIII = susceptible to 2/9 antibiotics tested; IX = susceptible to 1/9 antibiotics tested and X = susceptible to 0/9 antibiotics tested.

Resistance genes tested[2]: *aac*A-*aph*D, *erm*A, *erm*B, *tet*O, *tet*M, *tet*K, *tet*L, *tet*W, *tet*S, *mec*A, *bla*Z, *van*A and *van*B.

[3]: $p < 0.05$ = statistically significant.

3.4 LAB strains selected

According to the results obtained from the antagonism and antimicrobial susceptibility tests, strains exhibiting high bioprotective potential against all indicator pathogens, susceptibility to at least 6 of the 9 antibiotics tested, and absence of resistance genes in their genomes (Table 4) were selected for further testing of antimicrobial activity in the milk matrix. The three selected strains belong to the genus *Lactobacillus*: 2 *L. plantarum* strains (177 and 272G) and 1 *L. rhamnosus* strain (52).

3.5 Statistical analysis

ANOVA indicated that the differences in inhibition zones among the isolates were statistically significant ($p < 0.05$), suggesting variability in the efficacy of isolates 52, 177, and 272G against the tested pathogens *E. coli* (F value = 10.56, $p = 0.015$) and *L. monocytogenes* (F value = 8.21, $p = 0.029$). The data suggest that isolates 177, 272G and 52 presented the largest (20.3 mm against *E. coli* and 25.0 mm against *L. monocytogenes*), intermediate and smallest inhibition zones against both pathogens. The standard deviation indicates that the variation in the efficacy of these LAB against *L. monocytogenes* is smaller (1.45 mm) than that against *E. coli* (2.44 mm), suggesting more consistent efficacy against *L. monocytogenes.*

3.6 Antagonistic activity in milk

The results of the antagonistic activity of the strains *L. rhamnosus* 52, *L. plantarum* 177, and *L. plantarum* 272G against the indicator pathogens *L. monocytogenes* ATCC 5779 and *E. coli* O157:H7 IAL1848 in the milk matrix are presented in Fig. 4. The pH of the pure milk (negative control) remained practically unchanged throughout the incubation period (21 days, pH 6.5-6.0), which is consistent with the absence of microbial growth in the negative control plate. The artificial inoculation of *L. monocytogenes* and *E. coli* in pure culture resulted in normal bacterial growth curves, with an exponential phase at the beginning of the assay and cell viabilities of 2.63×10^8 and 0.3×10^4 CFU/mL, respectively, at the end of the experiment (21 days). The artificial inoculation of *L. monocytogenes* slightly altered the pH, which was similar to that of the negative control, and did not change the visual characteristics of the milk. On the other hand, *E. coli* in pure culture slightly reduced the pH, which stabilized at 5 at 48 hours and remained stable until 21 days. In that case, the visual characteristics of the milk were altered, resulting in coagulation/flocculation.

In the coculture with the artificial inoculation of 10^6 CFU/mL of each LAB and each indicator bacteria in milk, LAB 52 eliminated both pathogens, *L. monocytogenes* and *E. coli*, by the 7th day of coculture and remained viable in the milk at the end of the experimental period (2.3×10^8 and 1.9×10^8 CFU/mL, respectively). LAB 272G and LAB 177 also eliminated *E. coli* by the 7th day, but eliminated *L. monocytogenes* only on the 14th and 21st days, respectively, and remained viable at the end of the experiment (2×10^6 and 3.3×10^6 CFU/mL, respectively). In the assay with a lower concentration of indicator bacteria (inoculation of 10^6 CFU/mL of each LAB and 10^4 CFU/mL of each pathogen), all LAB eliminated *E. coli* by the 7th day. However, unlike the first assay, LAB 272G and LAB 177 eliminated *L. monocytogenes* on the 7th and 14th days, respectively. LAB 52 rapidly reduced the pH (within 48 hours) in both coculture assays, stabilizing at 3 from 48 hours with *E. coli* and from 14 days with *L. monocytogenes*. At 21 days of the assay, the milk inoculated with LAB 52 and 177 and the indicator pathogens presented a pH of 3, whereas the milk inoculated with LAB 272G and the pathogens presented a pH of 3.5.

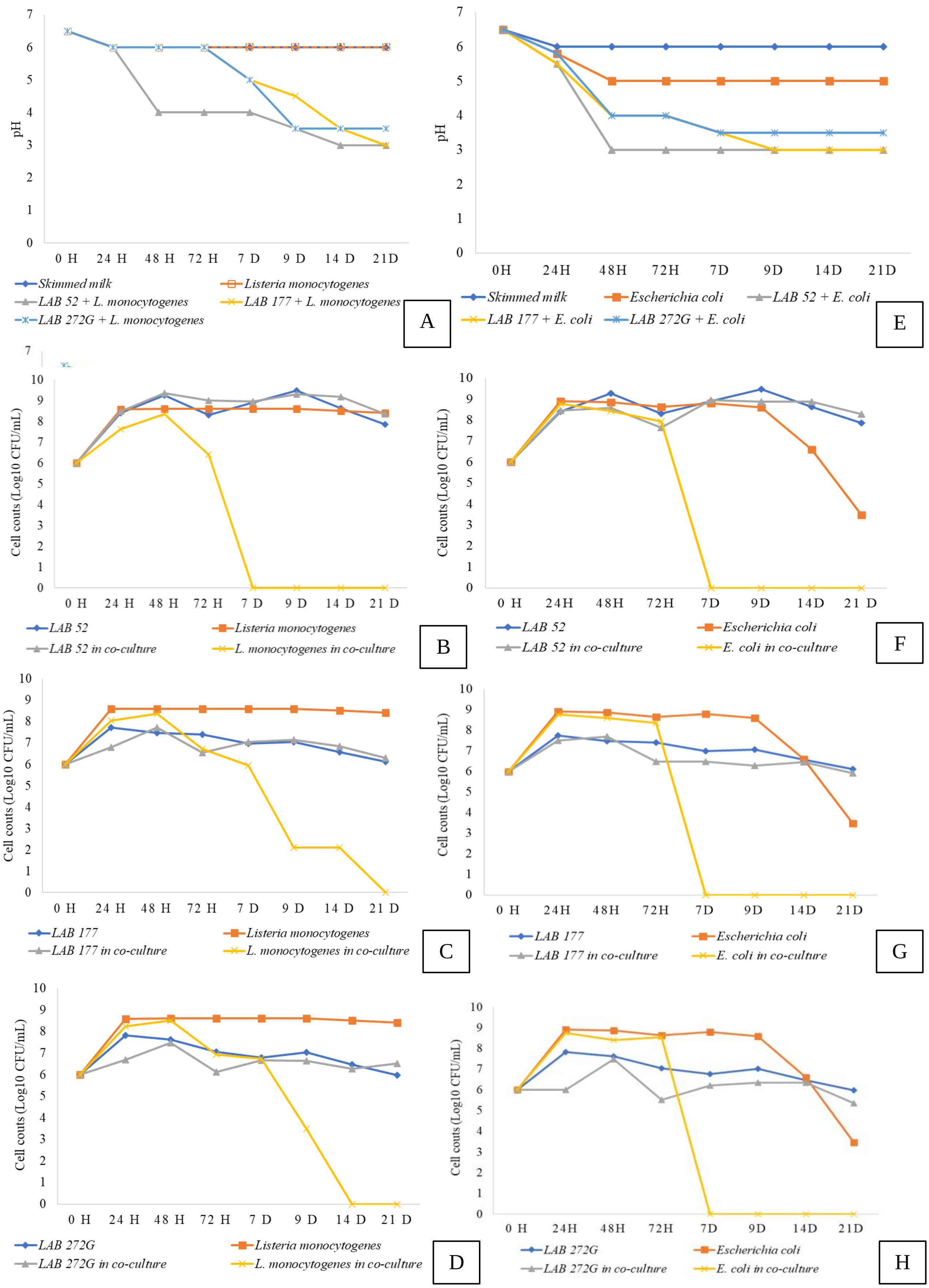

pH
0 H 24 H 48 H 72 H 7 D 9 D 14 D 21D
Skimmed milk
Listeria monocytogenes
LAB 52 + L. monocytogenes
LAB 177 + L. monocytogenes
LAB 272G + L. monocytogenes
A
pH
0H 24H 48H 72H 7D 9D 14D 21D
Skimmed milk
Escherichia coli
LAB 52 + E. coli
LAB 177 + E. coli
LAB 272G + E. coli
E
Cell couts (Log10 CFU/mL)
0 H 24 H 48 H 72 H 7 D 9 D 14 D 21 D
LAB 52
Listeria monocytogenes
LAB 52 in co-culture
L. monocytogenes in co-culture
B
Cell couts (Log10 CFU/mL)
0 H 24H 48H 72H 7D 9D 14D 21 D
LAB 52
Escherichia coli
LAB 52 in co-culture
E. coli in co-culture
F
Cell couts (Log10 CFU/mL)
0 H 24 H 48 H 72 H 7 D 9 D 14 D 21 D
LAB 177
Listeria monocytogenes
LAB 177 in co-culture
L. monocytogenes in co-culture
C
Cell couts (Log10 CFU/mL)
0 H 24H 48H 72H 7D 9D 14D 21D
LAB 177
Escherichia coli
LAB 177 in co-culture
E. coli in co-culture
G
Cell couts (Log10 CFU/mL)
0 H 24 H 48 H 72 H 7 D 9 D 14 D 21 D
LAB 272G
Listeria monocytogenes
LAB 272G in co-culture
L. monocytogenes in co-culture
D
Cell couts (Log10 CFU/mL)
0 H 24H 48H 72H 7D 9D 14D 21D
LAB 272G
Escherichia coli
LAB 272G in co-culture
E. coli in co-culture
H

Fig. 4. Validation of antagonistic activity in milk. **A, E:** Changes in the pH values of sterile skim milk artificially inoculated with pure cultures of *Listeria monocytogenes* ATCC 5779 and *Escherichia coli* O157:H7 IAL1848, respectively, and in coculture with *L. rhamnosus* 52, *L. plantarum* 177, and *L. plantarum* 272G at different time points: 0, 24, 48, and 72 hours and 7, 9, 14, and 21 days. **B - D and G - H:** Cell counts of *L. monocytogenes* ATCC 5779, *Escherichia coli* O157:H7 IAL1848, *L. rhamnosus* 52, *L. plantarum* 177, and *L. plantarum* 272G in sterile skim milk in pure cultures (10^6 CFU/mL) and in cocultures (10^6 CFU/mL of each pathogen + 10^6 CFU/mL of each LAB) at the same time points.

4. DISCUSSION

Among the 291 isolates initially identified as LAB from MAC samples, 84 genetically distinct strains were chosen to construct a portfolio of LAB to rationally prospect safe specimens with bioprotective properties against key pathogens and contaminants commonly associated with artisanal cheese production. The screening of isolates through rep-PCR allowed the exploitation of microbial genetic resources to be optimized, avoiding omission or redundancy of isolates through the choice of strains with distinct genetic profiles, enabling the establishment of a diverse microorganism collection for the purpose of the present study and for future research. The application of autochthonous LAB as bioprotective cultures in food requires a detailed screening process, including taxonomic identification at the species and strain levels, evidence of the inhibition of undesirable microorganisms, and susceptibility to antibiotics, which are among the essential criteria to ensure efficacy and safety (Bintsis & Papademas, 2024).

Most of the strains identified in the present study are enterococci or lactobacilli, with few representatives from other genera. The LAB species detected have been previously reported in MAC samples from the analyzed regions, though in varying proportions (Castro et al., 2016; Ferreira et al., 2022; Luiz et al., 2017; Margalho et al., 2020; Nero et al., 2021; Perin et al., 2017). Lactobacillus species, frequently predominant in these samples, are valued for their technological potential, safety, and versatility in food applications, especially in the dairy sector (Margalho et al., 2020; Nero et al., 2021; Souza et al., 2022). These LAB strains play a vital role in cheese maturation and enhance sensory attributes (Luiz et al., 2017). In this study, *L. plantarum* and *L. rhamnosus* were the most common lactobacilli species, which are frequently isolated from cheeses in the Araxá, Canastra, and Serro regions (Margalho et al., 2020; Perin et al., 2017; Silva et al., 2019).

Despite concerns regarding the potential pathogenicity and ability of *Enterococcus* spp. to transfer antimicrobial resistance genes (Fugaban et al., 2021; Schittler et al., 2019), food-derived strains, particularly *E. faecium*, generally exhibit lower pathogenic potential than clinical strains (Hugas et al., 2003). Enterococci species play a key role in enhancing the organoleptic properties of artisanal cheeses by improving aroma and flavor (Dapkevicius et al., 2021) and can inhibit foodborne pathogens such as *L. monocytogenes* (Silvetti et al., 2014; Zommiti et al., 2018). Therefore, assessing their virulence and susceptibility, especially to antimicrobials such as vancomycin, is crucial for evaluating their potential as bioprotective cultures (Castro et al., 2016). While only *E. faecium* is approved as a probiotic microorganism in foods for human consumption by Brazilian legislation (National Health Surveillance Agency [ANVISA], 2008), well-characterized and highly selected strains of other *Enterococcus* species may serve as valuable sources of bioactive molecules, offering potential for use as bioinputs in the dairy industry.

Owing to its nutritional richness, MAC serves as an ideal medium not only for the growth of LAB but also for the growth of undesirable microorganisms, including pathogens and spoilage agents. Microbiological assessments of MAC have revealed the presence of several foodborne pathogens, such as *L. monocytogenes* (Allaion et al., 2021; Firmo et al., 2023), *E. coli* O157:H7 (Carvalho et al., 2014), *Brucella abortus* (Silva et al., 2022), *Coxiella burnetii* (Rozental et al., 2020), and enteric viruses (norovirus and human adenovirus - HAdV) (Silva et al., 2021). Although the consumption of raw milk cheese in Brazil presents certain risks, this practice remains widespread, primarily due to the impact of heating during production on flavor and consumer acceptance of artisanal products. In this context, the use of LAB with bioprotective properties could significantly contribute to the sustainability of the cheese industry and the promotion of consumer health. Specifically, for MAC, current regulations prohibit the addition of external bacterial cultures or additives, as each cheese from its respective producer region is protected to preserve its unique characteristics. However, bioprotective cultures isolated from MAC itself could be employed in the development of new products, enhancing food safety. Moreover, making bioprotective strains isolated from MAC available could provide a basis for regulatory approval, supporting the inclusion of these strains in cheese from their region of origin and improving safety without compromising the authenticity of the product.

To our knowledge, this is the first study to evaluate the antagonism of LAB from MAC against *E. coli* O157:H7 and *E. faecalis*. In general, we observed that antagonism was more pronounced among *Lactobacillus* spp. strains against *L. monocytogenes* and *E. coli*, a trend not observed in strains of other LAB genera studied, with the exception of one strain of *E. mundtii*, which demonstrated high activity against *E. coli*. The strongest antagonistic potential was exhibited by *L. plantarum* against *E. coli* and *Listeria*, followed by *L. rhamnosus* against the same pathogens. Rodríguez-Sánchez et al. (2021) also reported antagonistic effects of *Lactobacillus* spp. strains against *L. monocytogenes*, reinforcing their role as bioprotective agents. In contrast, none of the 84 analyzed strains exhibited a halo/colony ratio greater than 3 against *E. faecalis*. The reduced antagonistic activity of LAB strains against *E. faecalis* can be attributed to the pathogen's inherent resistance to antimicrobial compounds produced by LAB, as these compounds often exhibit strain-specific activity (Tremonte et al., 2017). Additionally, in this study and other unpublished data from our group, we observed that the indicator strain *E. faecalis* ATCC 29212 demonstrates significant resistance to LAB antagonism. Based on these findings, we do not recommend the use of this strain for identifying LAB strains with antagonistic activity, as its high resistance may limit the detection of potential candidates with inhibitory properties.

The preservative effect of LAB is attributed mainly to the rapid acidification of raw materials through lactic acid production, which is likely responsible for the antagonistic activity observed in this study. Lactic acid exerts bacteriostatic or bactericidal effects on sensitive microorganisms (Grajek et al., 2005). Additionally, bioprotective cultures can produce other antimicrobial compounds, including organic acids, hydrogen peroxide, acetoin, diacetyl, reuterin, and antimicrobial peptides (Souza et al., 2022). Bacteriocins are ribosomally synthesized antimicrobial peptides with species-specific or broad-spectrum activity, and bacteriocin-producing bacteria are immune to their own bacteriocins (Cotter et al., 2005).

Under the conditions tested in this study, none of the 84 LAB strains produced protein-based molecules responsible for the inhibitory action against the indicator pathogens, as assessed by the activity of proteases proteinase K and pepsin. This suggests that bacteriocins were not responsible for the observed inhibitory effects. Bruno et al. (2017) also identified antagonism in lactobacilli strains isolated from artisanal cheeses against *L. monocytogenes*, *E. coli, Staphylococcus aureus,* and *Salmonella* spp. through the formation of inhibition halos around the spots of LAB supernatants. However, upon evaluating the nature of the antibacterial effect against these pathogens, they reported that it was attributed primarily to the acidic pH of the LAB supernatants.

LAB susceptibility to a broader range of antimicrobials, particularly to different classes, is essential for their potential use as bioinputs, as it minimizes the risk of transferring antibiotic resistance genes to human pathogens (Souza et al., 2007). In this study, most of the LAB strains were susceptible to at least 6 out of the 9 antibiotics tested. Most strains were resistant to gentamicin, cotrimoxazole, and streptomycin. The observed antibiotic susceptibility patterns align with those of previous studies (Charteris et al., 1998; Colombo et al., 2020), enabling the preselection of LAB strains with bioprotective potential and safe antibiotic profiles for use in food.

In antimicrobial resistance assessments, distinguishing between intrinsic and acquired resistance is essential. Intrinsic resistance is a natural characteristic of bacterial species or genera and poses a minimal risk of horizontal transfer. In contrast, acquired resistance, which emerges in previously susceptible strains, has high potential for horizontal transmission (Li et al., 2020). In addition to vancomycin, *Lactobacillus* spp. exhibit intrinsic resistance to gentamicin and streptomycin due to the low permeability of the cell surface to aminoglycosides (Anisimova & Yarullina, 2019; Charteris et al., 1998; Margalho et al., 2020). Enterococci strains are intrinsically resistant to cephalosporins, cotrimoxazole, and lincomycin and show low-level resistance to penicillin and aminoglycosides (Pandova et al., 2024). Vancomycin resistance is a critical safety marker for LAB, as it is used as a last-resort treatment for resistant infections such as MRSA (Al Atya et al., 2015; Chen, 2019). While vancomycin resistance is intrinsic to certain genera (Colombo et al., 2020), strains with this trait should be excluded from industrial use. Ruiz-moyano et al. (2019) similarly excluded *Enterococcus* strains with acquired resistance from further probiotic testing.

The presence of antimicrobial resistance genes in LAB raises concerns due to potential risks to human health, food safety, and the efficacy of antibiotic treatments. In food production, lactic acid cultures must be free of genes that can be transferred horizontally to other bacteria, including commensal and pathogenic species (Li et al., 2020). In the present study, the resistance genes *aac*A-*aph*D, *erm*A, *tet*M, and *tet*S were detected in some strains, and their presence served as a noncompliance criterion for selecting the top strains. Although *L. plantarum* strain 274 showed excellent antagonistic activity and gentamicin susceptibility, it was excluded because of the presence of the *aac*A-*aph*D gene, which encodes a bifunctional enzyme conferring broad resistance to nearly all aminoglycosides except streptomycin (Ramirez & Tolmasky, 2010). This gene is often located on transposons, such as Tn 4001, suggesting high transmissibility potential (Zhang et al., 2018).

Importantly, the functionality of antibiotic resistance genes, such as *aac*A-*aph*D, can be compromised by factors such as premature stop codons, insertions, or deletions, which may prevent the production of a functionally active protein. Therefore, the presence of a resistance gene does not necessarily indicate a microorganism's ability to resist the corresponding antibiotics (Colautti et al., 2022). Gene editing could be a potential solution for LAB strains carrying such genes but with important technological applications. In this study, despite evidence suggesting inactivity of the *aac*A-*aph*D gene in the *L. plantarum* strain 274, priority was given to selecting other bioprotective strains without resistance genes, owing to the risks their presence could pose for the intended applications of LAB strains.

Following the established selection criteria, three *Lactobacillus* strains were identified as superior: *L. rhamnosus* strain 52 and *L. plantarum* strains 177 and 272G. These strains demonstrated strong antagonistic activity against the indicator pathogens, were susceptible to at least six out of nine antibiotics tested, and had no resistance genes detected in their genomes. However, these strains were resistant to penicillin (*L. rhamnosus* 52, *L. plantarum* 177, and 272G), tetracycline (*L. rhamnosus* 52 and *L. plantarum* 177), and vancomycin (*L. plantarum* 177 and 272G) in disc diffusion tests. Further research using additional resistance primers is needed to investigate the genetic mechanisms behind these resistances. The genomes of these selected strains are already being sequenced and will be published elsewhere.

When determining the bioprotective properties of LAB, there is not always consistency between the results of in vitro and *in situ* or *in vivo* studies (Morales et al., 2020). Therefore, it is essential to assess the inhibitory activity of strains directly in food. Limited information is available regarding the antilisterial activity and efficacy of *Lactobacillus* spp. in controlling Gram-negative pathogens in milk (Aljasir et al., 2020; Iulietto et al., 2018). Furthermore, to our knowledge, this is the first in situ study evaluating the potential of lactobacilli strains from the MAC in the Araxa and Serro regions against important pathogens related to this artisanal product.

In the *in situ* assays, *L. rhamnosus* 52, *L. plantarum* 177, and L. plantarum 272G were effective at eliminating *L. monocytogenes* and *E. coli* in milk and remained at high counts for up to 21 days post inoculation. This finding aligns with previous research showing the efficacy of LAB in inhibiting pathogens in various food products (Ibrahim et al., 2021). Natural additives, including LAB, have proven effective against a broad range of pathogens, supporting their use in biopreservation (Muthuvelu et al., 2023). pH monitoring during the experiment revealed a significant influence of LAB on food conditions, with *L. rhamnosus* 52 causing a rapid pH drop to approximately 3, indicative of organic acid production that aids in pathogen inhibition.

The study revealed that *L. rhamnosus* 52 was particularly effective, eliminating both *L. monocytogenes* and *E. coli* by the 7th day of coculture and maintaining high viability in milk. The other strains, *L. plantarum* 177 and 272G, also demonstrated significant antimicrobial activity in milk. These findings underscore the potential of these strains as bioprotective cultures in MAC and corroborate previous research (Arena et al., 2016). Given that these strains originate from this specific food matrix, their adaptation to the environmental conditions of the product is likely to be more effective. This inherent suitability enhances their ability to inhibit the growth of pathogens and spoilage organisms, thereby extending shelf life and improving food safety. Moreover, their natural occurrence in the milk matrix reduces the likelihood of adverse sensory changes, which is crucial for maintaining product quality and consumer acceptance (Coelho et al., 2022).

L. monocytogenes is known for its resilience in various food matrices, including dairy products, and can survive and grow at refrigeration temperatures, posing a significant hazard in the food industry (Jordan et al., 2015). In the present study, *L. monocytogenes* remained viable in milk for at least 21 days when cocultured with *L. plantarum* 177 and *L. plantarum* 272G, although it was eventually eliminated. This extended survival highlights the robustness of the pathogen and underscores the need for effective biopreservation strategies. The persistence of viable *L. monocytogenes* in milk despite the presence of LAB strains suggests that while LAB can significantly reduce pathogen levels, complete elimination may be gradual and influenced by specific factors such as the LAB strain used, and the initial pathogen load.

As highlighted by Granato et al. (2010) and Terpou et al. (2019), there is a growing demand for functional and safe foods, underscoring the importance of probiotics in food formulations. The validation of LAB in real food matrices is essential for meeting food safety regulations and gaining approval from regulatory agencies (EFSA, 2017). Additionally, effectively communicating these findings to consumers can increase confidence in fermented dairy products by emphasizing their health and safety benefits.

The data obtained in this study are important for the food industry, enabling the development of effective pathogen control strategies through the use of LAB cultures and facilitating the creation of new fermented dairy products with probiotic and biopreservative properties. As an essential component in determining the bioprotective potential of these strains, studies are being conducted and will continue to be carried out by the research team, through the exploration of genomic data to complement the evaluation of safety and probiotic potential.

5. CONCLUSION

This study underscores the potential of LAB strains isolated from MAC as effective biopreservative agents in the food industry. The demonstrated inhibitory activity against important pathogens, such as *E. coli* O157:H7 and *L. monocytogenes*, highlights the functional promise of these microorganisms. Importantly, the identification of three lactobacilli strains (52, 177, and 272G) that combine robust antagonistic activity with antibiotic susceptibility strengthens their suitability for safe application in dairy products.

The findings provide a valuable foundation for developing safer and healthier food formulations, emphasizing the dual role of LAB in biopreservation and health promotion. Future research should focus on expanding the application of these strains across diverse food matrices and exploring their efficacy against other hazardous microorganisms relevant to the dairy supply chain.

CRediT authorship contribution statement

Joice Fátima Moreira Silva: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Bruna Vieira Alonso:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Paula Aparecida Azevedo Almeida:** Writing – original draft, Methodology, Investigation, Formal analysis, Conceptualization. **Isabela Vieira Barbosa:** Writing – original draft, Investigation. **Otávio Augusto Braga de Paula:** Writing – original draft, Investigation. **Letícia Ribeiro Barbosa:** Review & editing. **Laura Maria Bruno:** Review & editing, Conceptualization, Resources. **Liliane Denize Miranda Menezes:** Resources, review & editing. **Márcio Roberto Silva:** Resources, Review & editing, Conceptualization. **Geraldo Márcio da Costa:** Review & editing. **Mirian Pereira Rodarte:** Review & editing, Conceptualization. **João Batista Ribeiro:** Writing – review & editing, Supervision, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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CHAPTER – 3: Potentially probiotic *Lactococcus* spp. strains isolated from raw milk for bovine mastitis control

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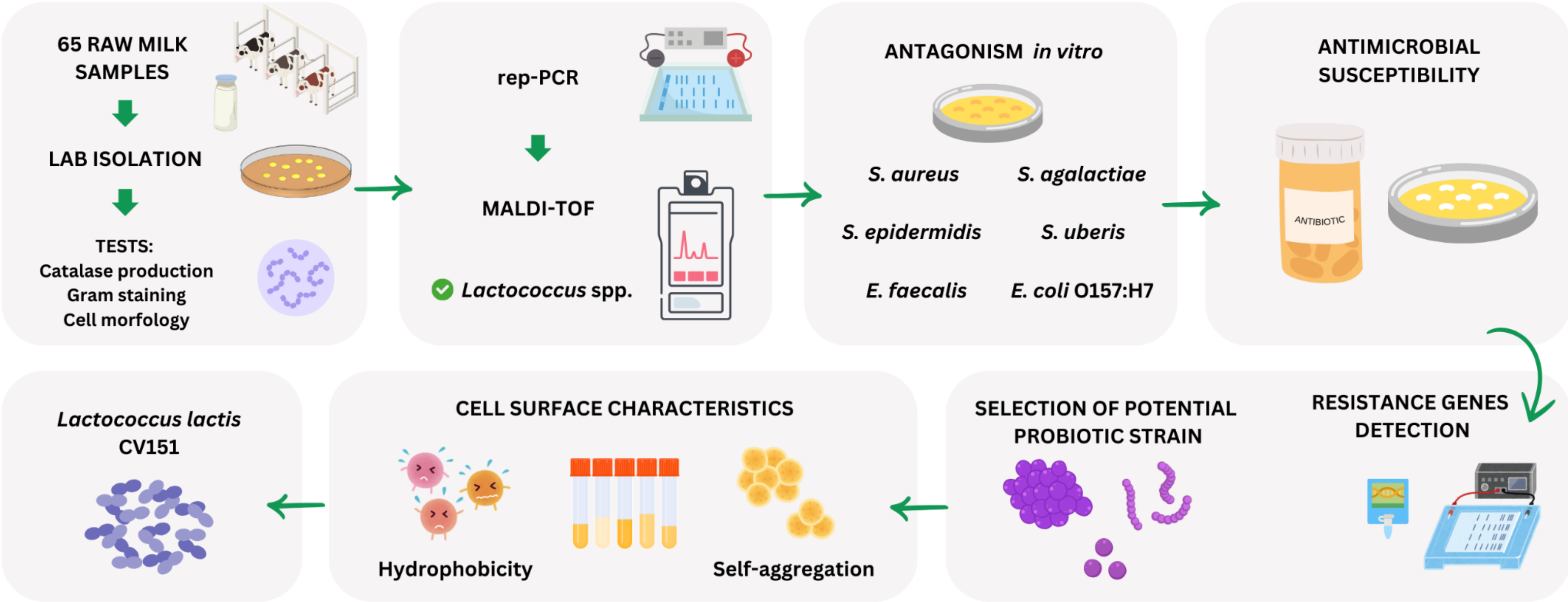
Abstract

Mastitis is one of the most prevalent diseases in dairy cows, causing significant economic losses in the milk production chain. Traditional control of the disease involves preventive and curative measures, including the use of antibiotics. However, the extensive use of these drugs has contributed to the selection of antimicrobial-resistant microorganisms, highlighting the need for natural alternatives for mastitis control. This study aimed to prospect *Lactococcus* spp. strains isolated from raw milk to evaluate their antagonistic effects *in vitro* against six bovine mastitis-causing pathogens and select potentially probiotic strains for the mastitis control. Raw milk samples from 65 dairy herds in Minas Gerais, Brazil, were screened for lactic acid bacteria (LAB), which were then evaluated genetically using rep-PCR and identified via MALDI-TOF. Nonredundant strains of *Lactococcus* spp. were tested for antagonistic effects against six mastitis-causing pathogens: *Staphylococcus aureus*, *Streptococcus agalactiae*, *Enterococcus faecalis*, *Escherichia coli* O157:H7, *Staphylococcus epidermidis*, and *Streptococcus uberis*. Additionally, the susceptibility of the strains to antimicrobials and the presence of resistance genes (*aac*A-*aph*D, *erm*B, *tet*O/M/K/S, *mec*A, *bla*Z and *van*A/B) were evaluated. Most *L. lactis* (*n* = 129) and *Lactococcus garvieae* (*n* = 18) strains exhibited good antagonistic activity, particularly against *S. aureus*, *S. uberis*, and *E. coli*. All the strains were susceptible to ampicillin and chloramphenicol, but demonstrated significant resistance to streptomycin (76%) and cotrimoxazole (96%). The resistance genes *blaZ* and *tetS* were detected in a small number of strains. Notably, *L. lactis* CV151 showed good antagonistic activity against all indicator pathogens, antimicrobial susceptibility, and absence of the investigated resistance genes, being a promising candidate for mastitis control. This study highlights the inhibitory effects and safety indications of *Lactococcus* spp. as potential alternative to traditional antibiotics for sustainable mastitis management.

Keywords: antagonism, bioprotection, *Escherichia coli,* lactic acid bacteria, *Staphylococcus aureus*.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Mastitis is the most commonly diagnosed disease in dairy cows and typically occurs as a result of intramammary infection by infectious agents such as bacteria, fungi, algae and viruses [1, 2]. Disease is characterized by physical, chemical, and biological changes in milk and mammary tissue due to inflammation, which reduce both the quality and quantity of the milk produced [3]. In addition to direct impacts on animal health, mastitis represents one of the main causes of economic losses in the dairy industry because of the costs of treatments, the disposal of contaminated milk, reduced production, and the premature culling of cows [4]. The total average cost attributed to bovine mastitis is estimated at $147 per cow per year, representing 11% to 18% of the annual gross margin of farms [5].

Clinical mastitis is characterized by visible signs of infection, such as changes in milk and the mammary gland, affecting approximately 25% of cows during each lactation period [2]. On the other hand, subclinical mastitis is characterized by the absence of visible changes in both milk and the mammary gland, but with an increased bulk milk somatic cell count (BMSCC) in response to the presence of the causative agent [6]. Damage to mammary tissue caused by subclinical mastitis leads to reduced milk production, accounting for 70% of total losses caused by this illness in dairy herds [7].

The control of mastitis traditionally involves a combination of preventive and curative measures [8]. Preventive measures include strict hygiene practices, proper herd management, and efficient milking programs to minimize cow exposure to pathogens [6]. Antibiotic therapy is commonly used both to treat acute infections and to prevent new infections during the dry period. Despite its effectiveness, the routine and indiscriminate use of antimicrobials has led to significant global health concerns, such as the increase in antibiotic resistance [9]. Considering the diverse strategies of genetic resistance among pathogens [10], and the zoonotic potential of some mastitis-causing pathogens [11], treating future infections in both animals and humans may become increasingly complex [12].

Given the challenges associated with antibiotic use, interest in natural alternatives for the prevention and control of bovine mastitis is increasing [8, 13, 14]. Methods such as genetic improvement for infection resistance [15, 16], the use of phytotherapeutics [17], herd vaccination [18], and the application of probiotics [19] are some of the strategies explored to reduce the incidence of the disease and to sustainably and safely reduce the use of antimicrobial agents. Among these alternatives, probiotics may offer superior applicability in terms of cost and effectiveness [13].

Probiotics are live microorganisms that provide several health benefits to the host when administered in adequate amounts, including the modulation of the intestinal microbiota, the strengthening of the immune system, and the inhibition of pathogen growth [20]. They are widely used in animal nutrition to improve animal health and performance [21]. Although the use of probiotics has proven effective in various applications in cattle farming, such as for the treatment of metritis [22], neonatal diarrhea [23], and the modulation of the digestive microbiome [24], their specific use in the control of bovine mastitis remains limited [14].

Among the various probiotic microorganisms, lactic acid bacteria (LAB) stand out due to their antimicrobial and immunomodulatory properties [21] and their recognition as safe (generally recognized as safe - GRAS) [25]. In the context of bovine mastitis, LAB can contribute to mammary gland health by reducing pathogen colonization and stimulating the local immune response through various mechanisms, such as adhesion to host epithelial cells, colonization, production of biosurfactants, auto-aggregation and co-aggregation with pathogens, production of antagonistic metabolites (organic acids, hydrogen peroxide, and bacteriocins), competition for nutrients, production of enzymes, and/or immunomodulation [8]. However, an important aspect to consider in the development of new probiotic products from LAB cultures for intramammary application is the adverse effects caused by potentially beneficial strains, such as the risk of tissue damage [26].

Lactococcus strains have shown significant probiotic potential and may be valuable tools in the prevention of mastitis in ruminants [19, 25 - 27]. Although *Lactococcus lactis* can be a potential pathogen of the mammary gland in dairy ruminants [28], the characteristics that distinguish dairy starter strains from mastitis-causing isolates—such as greater tolerance to body temperature, greater carbohydrate fermentation capacity, and better adhesion to mammary epithelial cells—are considered desirable probiotic attributes [14]. Moreover, these probiotic characteristics are strain-dependent, as their efficacy is primarily host-specific, making them more effective when isolated from their natural habitat, such as the raw milk [29].

Therefore, screening LAB to identify probiotic *Lactococcus* strains adapted to Brazilian herds is essential, as the use of autochthonous strains adapted to local conditions may increase the effectiveness of treatments and improve overall animal health. This process involves selecting strains with properties like pathogen inhibition, epithelial adhesion, antimicrobial production, immune modulation, and resilience to adverse conditions. Thus, the present study aimed to prospect *Lactococcus* spp. strains isolated from raw milk, assess their antagonistic potential *in vitro* against mastitis pathogens, and to select strains with promising probiotic properties for the control of bovine mastitis.

2. MATERIAL AND METHODS

2.1 Collection of raw milk samples

Raw milk samples from 65 dairy herds across 14 municipalities located in the Campo das Vertentes and South of Minas Gerais regions were aseptically collected from milk cooling tanks into sterile 40 mL vials. The samples were frozen with 10% glycerol to preserve their integrity during transport and stored at -20 °C until use.

2.2 Isolation of lactic acid bacteria (LAB)

The milk samples were thawed for bacterial isolation at room temperature. A 1 mL aliquot from each sample was serially diluted in phosphate-buffered solution (pH 6.2) (Merck, Germany) from 10^{-1} to 10^{-3}. Then, 100 μL of each dilution was plated on Petri dishes containing MRS agar (BD Difco, USA) and M17 agar (Sigma–Aldrich, USA). The plates were incubated at 35 °C for 24 to 48 hours. Phenotypically distinct bacterial colonies were selected and streaked for purification on MRS or M17 agar, according to the medium used for initial isolation, and the plates were incubated at 35 °C for 24 h. Subsequently, the isolates were tested for catalase production, subjected to Gram staining, and examined microscopically to determine cell morphology. All catalase-negative and Gram-positive cocci and rods were presumptively classified as LAB. The isolates were then transferred to microtubes containing milk with 10% glycerol and stored at -20 °C and -80 °C for further characterization.

2.3 Molecular typing

To assess genetic similarity and exclude duplicates, the LAB isolates were grouped by repetitive extragenic palindromic PCR (rep-PCR) following the method of Versalovic et al. [30]. Rep-PCR was performed using the universal primer (GTG)5 (5'-GTG GTG GTG GTG GTG-3'). Band patterns were analyzed using the Bionumerics software (version 6.6, Applied Maths, Sint-Martens-Latem, Belgium) with the Jaccard coefficient.

2.4 MALDI-TOF identification

The taxonomic identification of genetically nonredundant LAB strains was performed by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF), following the procedure proposed by Nacef et al. [31]. Protein profiles were obtained from bacterial cultures via ethanol/formic acid extraction. Bacterial suspensions were processed through a series of washing, ethanol inactivation, air-drying, and protein extraction steps using formic acid and acetonitrile. Extracted proteins were applied to a MALDI plate, coated with α-cyano-4-hydroxycinnamic acid matrix, and analyzed with an Autoflex III SmartBeam mass spectrometer (Bruker Daltonics). Mass spectra were generated in positive linear mode, with external calibration using the Bacterial Test Standard calibration mix (Bruker Daltonics). Identification scores between 3.0 and 2.0 were considered high-confidence identifications; scores between 1.70 and 1.99 were considered medium confidence; and scores below 1.69 were considered nonidentifiable. A standard *Escherichia coli* strain was used as a control.

2.5 Selection criteria for LAB strains

For the purposes of this study, among the genetically distinct LAB strains identified at the genus and species level by MALDI-TOF, *Lactococcus* spp. strains were selected to evaluate their antagonistic activity against mastitis pathogens *in vitro* and their potential use as probiotics to control the disease.

2.6 Antagonistic activity of *Lactococcus* spp. against bovine mastitis pathogens

The antagonistic activity of nonredundant *Lactococcus* spp. strains was assessed using the spot-on-lawn technique, as proposed by Tagg et al. [32], with modifications. Six mastitis-causing pathogens were used as indicator bacteria: *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus uberis* ATCC 700407. LAB strains were grown on MRS agar (35 °C/24 h). After growth, three colonies of each LAB strain were transferred to 1,000 µL of MRS broth and incubated at 35 °C ± 2 °C for 24 h. Then, 5 µL of each bacterial suspension were spotted at five equidistant points on MRS agar. The plates were incubated aerobically at 35 ± 2 °C for 24 h. In parallel, the indicator bacteria were inoculated into 10 mL of BHI broth and incubated at 37 °C for 4 h. Subsequently, 100 µL of each pathogen culture in suspension were added to 7 mL of prewarmed BHI semisolid agar (50 °C), and this mixture was poured over the MRS plate, forming a layer over the LAB spots. The Petri dishes were reincubated aerobically for 14 to 18 h at 35 °C ± 2 °C. The presence of a clear zone, measured in millimeters (mm) around the LAB spots/colonies, was considered evidence of antibacterial activity against the indicator pathogen [33]. The ratio between the size of the inhibition zone (mm) and the size of the colony or spot (mm) was used in this study to determine antagonistic activity and classify the strains based on the antagonistic effect, as the available literature criteria are subjective and not standardized. LAB strains with a halo/colony ratio less than 1 were classified as nonantagonistic (-); those with a halo/colony ratio between 1 and 2 were considered to have regular antagonistic activity (+); those with a halo/colony ratio between 2 and 3 were considered to have good antagonistic activity (++); and LAB strains with a halo/colony ratio greater than 3 were categorized as having excellent antagonistic activity (+++).

2.7 Assessment of the chemical nature of the inhibitory substances

The methodology proposed by Lewus and Montville [33] and adapted by Santos et al. [34] was used to evaluate the sensitivity of inhibitory substances produced by LAB to proteases and amylase. The same procedure described in the previous section was repeated, with some modifications. Before the BHI semisolid agar was poured over the Petri dish, 5 μL of an aqueous solution of proteinase K (20 mg/mL), amylase (20 mg/mL), and pepsin (20 mg/mL) were added next to the LAB spots/colonies. The plates were incubated at 35 °C for another two hours. Subsequently, 7 mL of BHI semisolid broth containing the indicator pathogen was poured over the same plates. The absence of an inhibition halo in the region where the protease was applied (in a half-moon shape) was considered indicative of the proteinaceous origin of the inhibitory substance. A control plate without the addition of enzymes was used as a negative control, and the test was performed in triplicate.

2.8 Selection criteria for *Lactococcus* spp. strains

Based on the results of the antagonism assay, *Lactococcus* spp. strains that exhibited good to excellent antagonistic activity, according to the halo/colony ratio, against at least 4 of the 6 tested indicator pathogens were selected for antimicrobial susceptibility characterization.

2.9 Assessment of antimicrobial susceptibility of *Lactococcus* spp.

Antimicrobial susceptibility testing was performed using the disk diffusion method proposed by Bauer et al. [35], with modifications made by Santos et al. [34]. *Lactococcus* spp. strains, selected as described in the previous section, were reactivated on MRS agar at 37 °C for 24 h to obtain isolated colonies. After growth, 3 colonies were collected and inoculated into MRS broth and incubated for 24 h at 37 °C. Subsequently, 100 μL of the culture were inoculated onto Petri dishes containing 25 mL of MRS agar and spread over the plate using a Drigalski loop. Then, 9 discs containing the following antimicrobials (Oxoid®, Basingstoke, England) were placed on the Petri dishes: penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), and cotrimoxazole (1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole). The plates were incubated aerobically for 24 h at 35 °C. To evaluate the results, the inhibition zones (mm) were measured with a millimeter ruler, and the strains were classified as sensitive or resistant to each antimicrobial, as proposed by Charteris et al. [36].

2.10 Detection of antibiotic resistance genes

The selected *Lactococcus* spp. strains were evaluated for the presence of the following antibiotic resistance genes commonly found in mastitis pathogens and pathogens potentially transmitted through milk and dairy products: *erm*A, *erm*B, *tet*O, *tet*M, *tet*K, *tet*L, *tet*W, *tet*S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A, and *van*B. The detection of antibiotic resistance genes was performed by PCR following protocols previously described in the literature (Table 1).

Table 1. Primers used to detect antibiotic resistance genes in the selected LAB.

Target gene	Primer	Amplicon size (pb)	Reference
*erm*B	*erm*B 1 - CGTTTACGAAATTGG*AAC*AGGTAAAGGGC *erm*B 2 - GAATCGAGACTTGAGTGTGC	359	Lina et al. [36]
*tet*O	*tet*O 1 - *AAC*TTAGGCATTCTGGCTCAC *tet*O 2 - TCCCACTGTTCCATATCGTCA	515	Ng et al. [37]
*tet*M	*tet*M 1 - AGTTTTAGCTCATGTTGATG *tet*M 2 - TCCGACTATTTAGACGACGG	1862	Trzcinski et al. [38]
*tet*K	*tet*K 1 - TATTTTGGCTTTGTATTCTTTCAT *tet*K 2 - GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al. [38]
*tet*L	*tet*L 1 - ATAAATTGTTTCGGGTCGGTAAT *tet*L 2 - *AAC*CAGCC*AAC*TAATGACAATGAT	1077	Trzcinski et al. [38]
*tet*W	*tet*W 1 - GAGAGCCTGCTATATGCCAGC *tet*W 2 - GGGCGTATCCACAATGTT*AAC*	168	Aminov et al. [39]
*tet*S	*tet*S 1 - TGG*AAC*GCCAGAGAGGTATT *tet*S 2 - ACATAGACAAGCCGTTGACC	740	Obioha et al. [40]
*aac*A-*aph*D	*aac*A-*aph*D 1 - TAATCCAAGAGCAATAAGGGC *aac*A-*aph*D 2 - GCCACACTATCAT*AAC*CACTA	228	Strommenger et al. [41]
*mec*A	*mec*A 1 - TGGCTATCGTGTCACAATCG *mec*A 2 - CTGG*AAC*TTGTTGAGCAGAG	310	Vannuffel et al. [42]
*bla*Z	*bla*Z 1 - AAGAGATTTGCCTATGCTTC *bla*Z 2 - GCTTGACCACTTTTATCAGC	517	Vesterholm-Nielsen et al. [43]
*van*A	*van*A 1 - TCTGCAATAGAGATAGCCGC *van*A 2 - GGAGTAGCTATCCCAGCATT	377	Lemcke et al. [45]
*van*B	*van*B 1 - GCTCCGCAGCCTGCATGGACA *van*B 2 - ACGATGCCGCCATCCTCCTGC	529	Lemcke et al. [45]

2.11 Growth at different temperatures

To verify the growth capacity at different temperatures, fresh and isolated colonies of each selected strain of *Lactococcus* spp. were inoculated into 1 mL of MRS broth and incubated at 35 °C for 12 h. Subsequently, 5 µL of the bacterial suspension were added at equidistant points on Petri dishes containing MRS agar, in triplicate. The plates were incubated at 35, 43, and 48 °C. After 48 hours, the presence (+) or absence (-) of bacterial growth at the inoculation points was recorded.

2.12 Selection criteria for potential probiotic *Lactococcus* spp. strain

For subsequent tests, the *Lactococcus* strain that met the following criteria was selected: antagonistic effects against all indicator pathogens, susceptibility to all 9 antibiotics tested, absence of antimicrobial resistance genes, and ability to grow at a minimum of 43 °C.

2.13 Assessment of cell surface characteristics

2.13.1 Hydrophobicity

To determine hydrophobicity, fresh and isolated colonies of the *Lactococcus* strain selected according to the criteria described in the previous section were grown three times in MRS broth at 35 °C for 12 h. The third culture was centrifuged at 2,000 × *g* for 10 min, and the supernatants were discarded. The resulting pellets were washed three times with sterile saline solution and resuspended in the same solution until an initial optical density (ODi) between 0.4 and 0.6 (at 600 nm) was achieved, following the microbial adhesion to hydrocarbons (MATH) method described by Otero et al. [46]. Next, 3.6 mL aliquots of the bacterial suspensions were distributed into sterile Falcon tubes, and 0.6 mL of hexadecane (Sigma, USA) were added to each tube. After shaking, the tubes were allowed to stand for 1 hour at 37 °C to allow separation of the immiscible solvent and the aqueous phase. After phase separation, the aqueous layer was removed and transferred to a clean tube for determination of the final absorbance (ODf). The hydrophobicity (%) was calculated using the equation below:

$$\% \text{ hydrophobicity} = [(ODi - ODf) / ODi] \times 100$$

ODi = initial optical density
ODf = final optical density

2.13.2 Self-aggregation

To assess self-aggregation ability, the selected *Lactococcus* spp. strains were subcultured three times in MRS broth at 35 °C for 12 h. The third culture was centrifuged at 2,000 × *g* for 15 min, and the supernatants were discarded. The pellets were washed three times with phosphate-buffered saline (PBS, pH 6.5) and resuspended in the same solution until an initial optical density (ODi) of 0.4 to 0.6 (600 nm) was reached, according to the methodology described by Ocaña and Nader-Macías [47], with modifications. The solutions were monitored spectrophotometrically for 4 hours, at 15-minute intervals, allowing sedimentation during the spectrophotometric determinations to obtain the final optical density (ODf). The percentage of self-aggregation was calculated using the following equation:

$$\% \text{ self-aggregation} = [(1 - \text{ODf}) / \text{ODi}] \times 100$$

ODi = initial optical density

ODf = final optical density

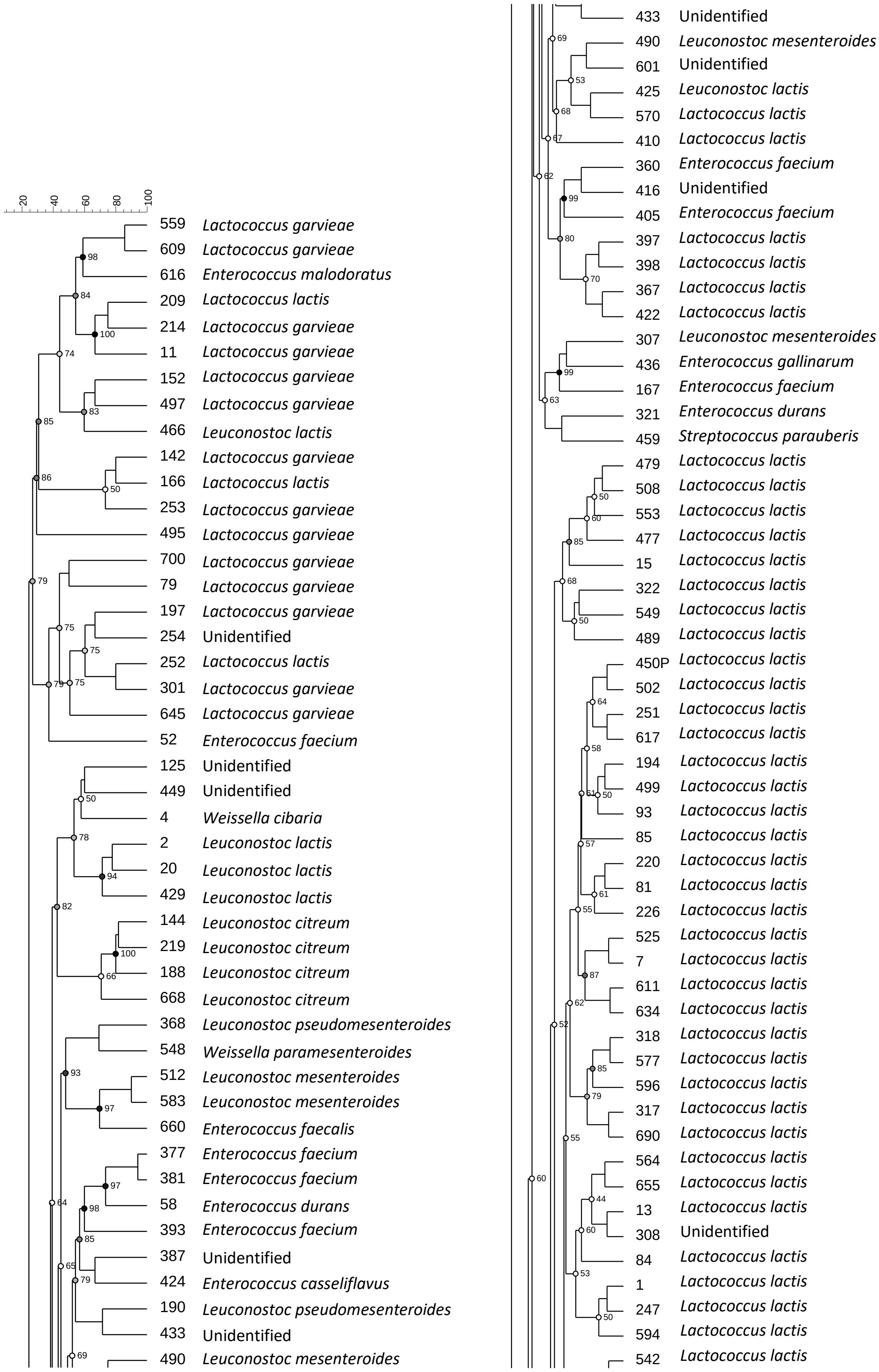
3. RESULTS

3.1 Isolation, genetic diversity, and identification of LAB

Raw milk samples collected from 65 dairy cattle herds were used to prospect for autochthonous LAB. Among the 701 microorganisms initially isolated, 368 were Gram-positive and catalase-negative, and therefore presumptively classified as LAB. Of these, 210 were isolated on MRS culture media and 158 on M17 media. Morphologically, 94 isolates were identified as cocci, 220 as coccobacilli, and 54 as bacilli.

In the evaluation of genetic similarity using the rep-PCR technique, 221 isolates were considered genetically distinct and subjected to identification by MALDI-TOF, which revealed the following species, as shown in the dendrogram in Figure 1: *Lactococcus lactis* (*n* = 129), *Lactococcus garvieae* (*n* = 18), *Enterococcus faecalis* (*n* = 23), *Enterococcus faecium* (*n* = 10), *Enterococcus durans* (*n* = 3), *Enterococcus casseliflavus* (*n* = 2), *Enterococcus italicus* (*n* = 2), *Enterococcus gallinarum* (*n* = 1), *Enterococcus malodoratus* (*n* = 1), *Leuconostoc lactis* (*n* = 6), *Leuconostoc citreum* (*n* = 5), *Leuconostoc mesenteroides* (*n* = 7), *Leuconostoc pseudomesenteroides* (*n* = 2), *Streptococcus parauberis* (*n* = 4), *Weissella cibaria* (*n* = 1), and *Weissella paramesenteroides* (*n* = 1). The MALDI-TOF identification score values ranged from 1.7 to 2.41, indicating medium to high confidence levels.

According to the selection criteria of the present study, 147 genetically distinct strains of the genus *Lactococcus* were chosen for characterization of their antagonistic and probiotic potential against bovine mastitis pathogens. The selected LAB strains belong to the following species: *L. lactis* (*n* = 129) and *L. garvieae* (*n* = 18) (Table 2). The genetic diversity of the isolates, confirmed by rep-PCR, allowed the formation of a collection of lactococci with distinct profiles for the evaluation of their potential application in controlling bovine mastitis, in accordance with the study's objective.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497 Lactococcus garvieae
466 Leuconostoc lactis
142 Lactococcus garvieae
166 Lactococcus lactis
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52 Enterococcus faecium
125 Unidentified
449 Unidentified
4 Weissella cibaria
2 Leuconostoc lactis
20 Leuconostoc lactis
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144 Leuconostoc citreum
219 Leuconostoc citreum
188 Leuconostoc citreum
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368 Leuconostoc pseudomesenteroides
548 Weissella paramesenteroides
512 Leuconostoc mesenteroides
583 Leuconostoc mesenteroides
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377 Enterococcus faecium
381 Enterococcus faecium
58 Enterococcus durans
393 Enterococcus faecium
387 Unidentified
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433 Unidentified
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436 Enterococcus gallinarum
167 Enterococcus faecium
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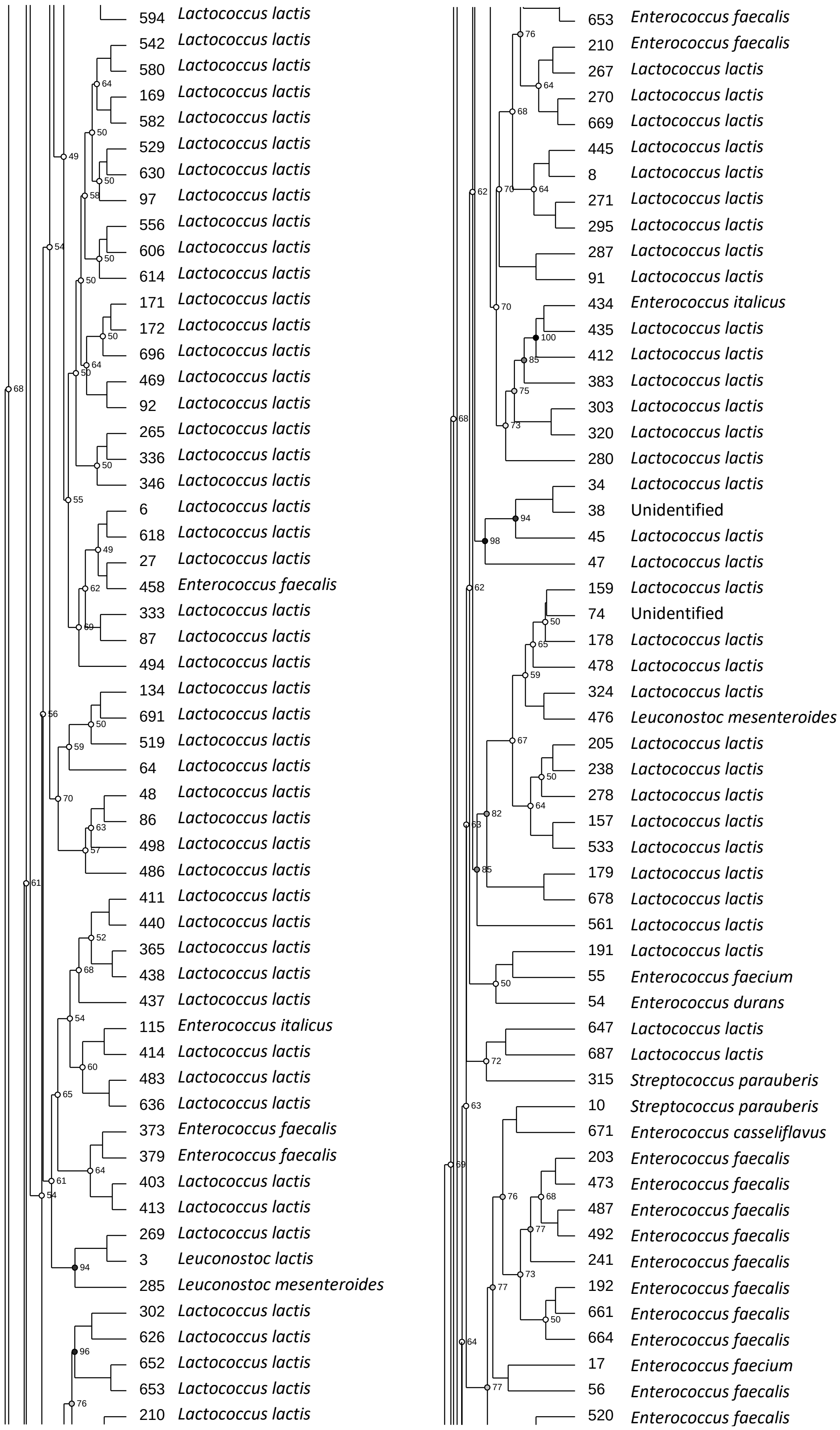
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606 Lactococcus lactis
614 Lactococcus lactis
171 Lactococcus lactis
172 Lactococcus lactis
696 Lactococcus lactis
469 Lactococcus lactis
92 Lactococcus lactis
265 Lactococcus lactis
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191 Lactococcus lactis
55 Enterococcus faecium
54 Enterococcus durans
647 Lactococcus lactis
687 Lactococcus lactis
315 Streptococcus parauberis
10 Streptococcus parauberis
671 Enterococcus casseliflavus
203 Enterococcus faecalis
473 Enterococcus faecalis
487 Enterococcus faecalis
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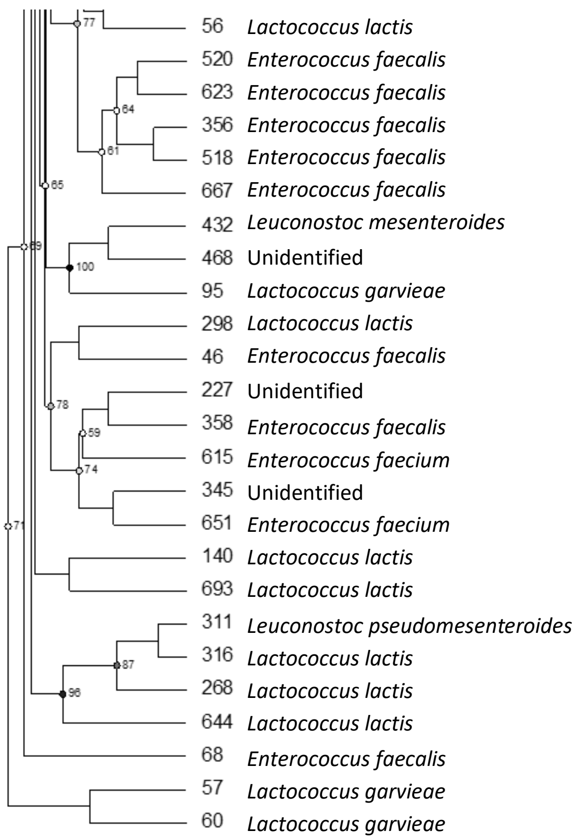

Figure 1. Dendrogram based on the cluster analysis of fingerprints by rep-PCR and identification of lactic acid bacteria (LAB) species using MALDI-TOF. Bionumerics software (version 6.6, Applied Maths, Sint-Martens-Latem, Belgium).

3.2 Antagonistic activity of *Lactococcus* spp. against pathogens causing bovine mastitis

The antagonistic capacity of genetically nonredundant *Lactococcus* spp. strains, determined by the spot-on-lawn technique, is shown in Figure 2 and Table 2. Overall, the strains exhibited the strongest antagonistic effects against the indicators *S. aureus* ATCC 25923, *S. uberis* ATCC 700407, and *E. coli* O157:H7 IAL1848.

In terms of species, 79% of the *L. lactis* strains showed good antagonistic activity against *S. aureus*, and 73.6% showed good antagonistic activity against *S. agalactiae* ATCC 12386. Additionally, six *L. lactis* strains (CV7, CV13, CV580, CV614, CV655, and CV691) had a halo/colony ratio greater than 3 for *S. aureus*, which is considered excellent antagonistic capacity by the criteria established in this study. *S. epidermidis* ATCC 12228, on the other hand, was the most resistant pathogen to *L. lactis*, showing no susceptibility to 14% of the tested strains. *L. garvieae* demonstrated moderate antagonistic activity against *S. agalactiae*, *S. epidermidis*, and *E. faecalis* ATCC 29212. For *S. aureus* and *E. coli*, 72% of the *L. garvieae* strains showed good antagonism. All *L. garvieae* strains demonstrated at least moderate antagonistic activity (halo/colony ratio > 1) against *S. uberis*, with only one strain (*L. garvieae* CV197) showing excellent antagonistic activity against this pathogen.

Individually, the *L. lactis* strains CV34 and CV151 stood out by exhibiting good antagonistic activity (halo/colony ratio > 2) against all tested indicator pathogens. Moreover, the *L. lactis* strain CV15 showed the greatest antagonistic effect in the entire assay (halo/colony ratio = 5), which occurred against the pathogen *S. uberis*.

Regarding the chemical nature of the inhibitory substances, no retraction or absence of inhibition halos was observed at the enzyme application sites of proteinase K, pepsin, or amylase for any of the 147 strains of *Lactococcus* spp.

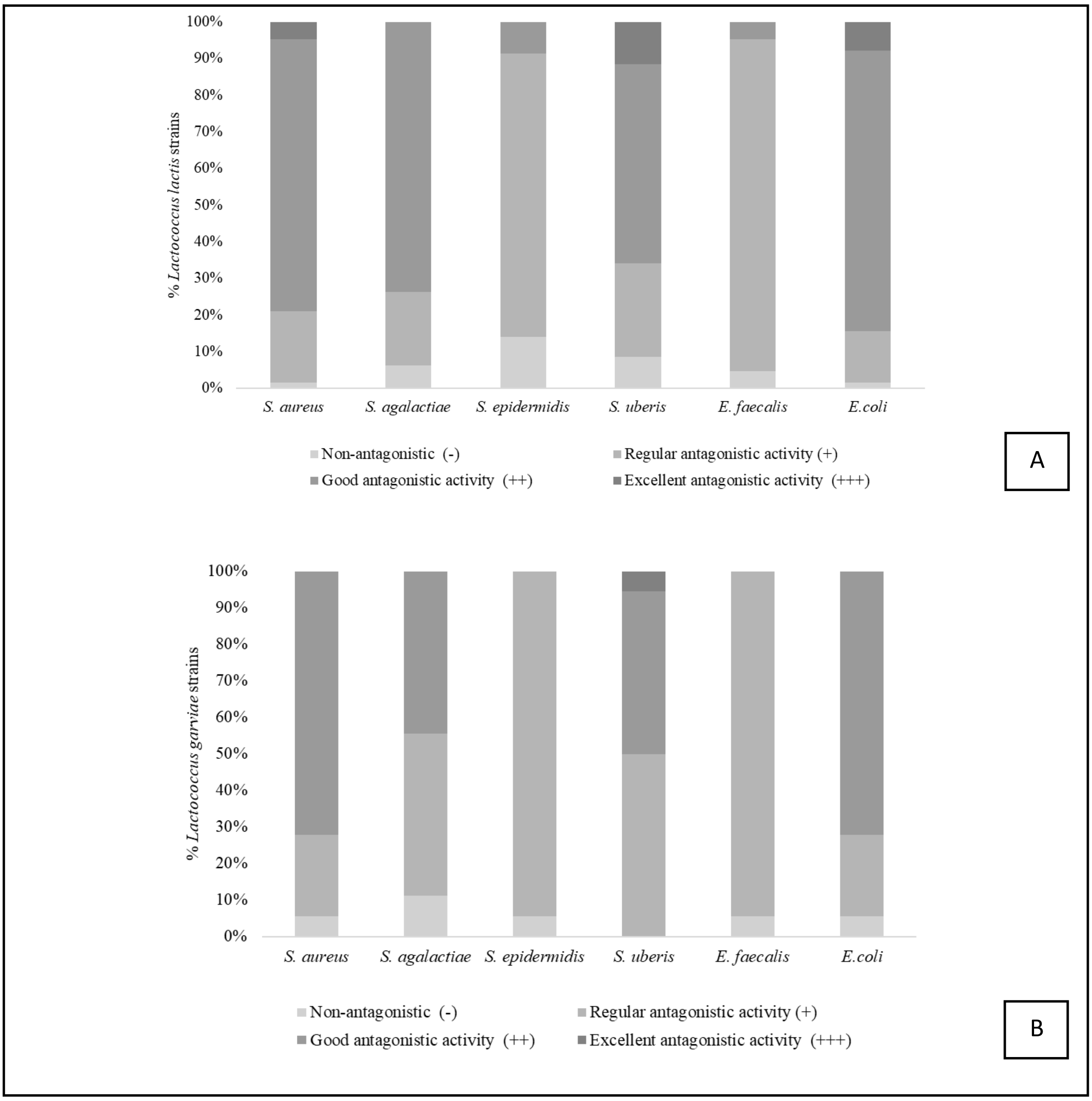

Figure 2. A: Antagonistic activity of *Lactococcus lactis* (*n* = 129) and *Lactococcus garvieae* (*n* = 18) **(B)** against pathogens causing bovine mastitis: *S. aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus uberis* ATCC 700407.

Table 2. Antagonistic activity (halo/colony ratio in mm) of *Lactococcus* spp. strains isolated from raw milk against pathogens causing bovine mastitis.

MALDI-TOF identification		**Indicator pathogens** **Mean halo/colony ratio followed by the range of values** [2]					
LAB species	*n*[1]	*Staphylococcus aureus* ATCC 25923	*Streptococcus agalactiae* ATCC 12386	*Staphylococcus epidermidis* ATCC 12228	*Streptococcus uberis* ATCC 700407	*Enterococcus faecalis* ATCC 29212	*Escherichia coli* O157:H7 IAL 1848
Lactococcus lactis	129	2.26 [0.0 - 3.50] (++)*	2.13 [0.0 - 2.94] (++)	1.44 [0.0 - 2.66] (+)	2.19 [0.0 - 5.0] (++)	1.60 [0.0 - 2.0] (+)	2.37 [0.0 - 3.57] (++)
Lactococcus garvieae	18	2.03. [0.0 - 2.64] (++)	1.72 [0.0 - 2.71] (+)	1.44 [0.0 - 1.83] (+)	2.13 [1.48 - 3.34] (++)	1.45 [0.0 - 1.80] (+)	2.10 [0.0 - 2.91] (++)
Total number of strains	147	2.21 [0.0 - 3.50] (++)	2.06 [0.0 - 2.94] (++)	1.43 [0.0 - 2.66] (+)	2.16 [0.0 - 5.0] (++)	1.56 [0.0 - 2.0] (+)	2.31 [0.0 - 3.57] (++)

[1]: Number of LAB strains isolated per species.

[2]: Halo/colony ratio = mean halo/colony ratio by species of *Lactococcus* spp., followed by the lowest and highest values in the range (in parentheses).

*Halo/colony ratio < 1 = nonantagonistic (-); halo/colony ratio > 1 = regular antagonistic activity (+); halo/colony ratio > 2 = good antagonistic activity (++); halo/colony ratio > 3 = excellent antagonistic activity (+++).

3.3 Susceptibility of *Lactococcus* spp. to antimicrobials and detection of resistance genes

According to the selection criteria of the present study, 24 strains of *Lactococcus* spp. that exhibited good to excellent antagonistic activity against at least 4 of the 6 indicator pathogens tested were selected for antimicrobial susceptibility evaluation. The susceptibility and antibiotic susceptibility profiles of the *L. lactis* strains (*n* = 23) and *L. garvieae* (*n* = 1), as well as the resistance genes detected, are detailed in Table 3. All evaluated *Lactococcus* strains were susceptible to ampicillin and chloramphenicol. Most strains were susceptible or moderately susceptible to penicillin (96%), erythromycin (92%), vancomycin (84%), tetracycline (76%), and gentamicin (56%). In contrast, most strains were resistant to streptomycin (76%), and nearly all were resistant to co-trimoxazole (96%).

The susceptibility profiles are expressed in terms of *Lactococcus* strains and their susceptibility to 9 of the 9 tested antibiotics (profiles I to X, respectively). All the strains were susceptible to at least 4 of the 9 antibiotics tested. The majority of the strains (52%) exhibited susceptibility to between 7 and 9 antibiotics (profiles III, II, and I, respectively). The *L. lactis* strain CV151 was susceptible to all the tested antibiotics (profile I).

The analysis of resistance genes revealed the presence of the *tetS* gene in five strains of *L. lactis* (CV252, CV271, CV519, CV521, and CV669), indicating potential resistance to tetracycline in these strains. Additionally, the *blaZ* gene, associated with beta-lactam resistance, was identified in a single strain, *L. lactis* CV252.

3.4 Growth at different temperatures

Regarding the growth of *Lactococcus* spp. at different temperatures after 48 hours of incubation, all the strains were grown at 35 °C (the isolation temperature), 21 were grown at 43 °C, and 11 were grown at 48 °C.

Table 3. Susceptibility of *Lactococcus* spp. strains to the antibiotics by the disk diffusion method, susceptibility profile, and detected resistance genes.

***Lactococcus* spp. strains**	***Lactococcus* strains susceptible to the antibiotics tested[1]**									**Susceptibility profile[2]**	**Resistance genes detected[3]**
	AMP	**PEN**	**CHL**	**SXT**	**ERI**	**STR**	**GEN**	**TET**	**VAN**		
Lactococcus lactis CV7	S	MS	S	R	S	S	S	S	S	II	
Lactococcus lactis CV13	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV15	S	S	S	R	S	R	R	R	S	V	
Lactococcus lactis CV27	S	MS	S	R	S	S	R	S	MS	III	
Lactococcus lactis CV34	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV94	S	S	S	R	S	S	S	S	S	II	
Lactococcus lactis CV97	S	MS	S	R	S	R	S	S	S	III	
Lactococcus lactis CV151	S	S	S	S	S	S	S	S	S	I	
Lactococcus lactis CV205	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV252	S	MS	S	R	R	R	R	R	S	VI	*bla*Z; *tet*S
Lactococcus lactis CV268	S	S	S	R	S	MS	S	S	R	III	
Lactococcus lactis CV269	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV271	S	S	S	R	S	R	R	R	R	VI	*tet*S
Lactococcus lactis CV434	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV483	S	S	S	R	S	R	R	S	S	IV	
Lactococcus lactis CV519	S	S	S	R	S	S	R	R	S	IV	*tet*S
Lactococcus lactis CV521	S	S	S	R	S	R	S	R	S	IV	*tet*S
Lactococcus lactis CV542	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV564	S	R	S	R	R	R	R	S	S	VI	
Lactococcus lactis CV580	S	MS	S	R	S	R	R	S	R	V	
Lactococcus lactis CV614	S	S	S	R	S	R	S	S	S	III	
Lactococcus lactis CV655	S	S	S	R	S	R	S	S	R	IV	

Lactococcus lactis CV669	S	MS	S	R	S	R	R	R	S	V	*tet*S
Lactococcus lactis CV691	S	MS	S	R	S	R	R	S	S	IV	
Lactococcus garvieae CV197	S	MS	S	R	S	R	R	S	S	IV	
Total susceptible or moderately susceptible (%)	100	96	100	4	92	24	56	76	84	-	-

S = susceptible; MS = moderately susceptible; R = resistant.

[1]: AMP = Ampicillin; PEN = Penicillin; CHL = Chloramphenicol; SXT = Cotrimoxazole; ERI = Erythromycin; STR = Streptomycin; GEN = Gentamicin; TET = Tetracycline; VAN = Vancomycin.

[2]: I = Susceptible to all antibiotics tested; II = Susceptible to 8/9 antibiotics tested; III = Susceptible to 7/9 antibiotics tested; IV = Susceptible to 6/9 antibiotics tested; V = Susceptible to 5/9 antibiotics tested; VI = Susceptible to 4/9 antibiotics tested; VII = Susceptible to 3/9 antibiotics tested; VIII = Susceptible to 2/9 antibiotics tested; IX = Susceptible to 1/9 antibiotics tested; X = Susceptible to 0/9 antibiotics tested.

[3] : Resistance genes tested: *aacA-aphD*, *ermB*, *tetO*, *tetM*, *tetK*, *tet*S, *tet*L, *tet*W, *mecA*, *blaZ*, *vanA*, and *vanB*.

3.5 Cell surface characteristics

The *L. lactis* strain CV151 was selected as the superior strain according to the selection criteria and was subjected to hydrophobicity and self-aggregation tests. As shown in Table 4, the strain exhibited low hydrophobicity (14.96%) and high self-aggregation capacity (84.03%).

Table 4. Probiotic properties of the *Lactococcus lactis* strain CV151.

Probiotic characteristics	***Lactococcus lactis* CV151**
Staphylococcus aureus ATCC 25923	2,85[1] (++)*
Streptococcus agalactiae ATCC 12386	2,76 (++)
Staphylococcus epidermidis ATCC 12228	2,00 (++)
Streptococcus uberis ATCC 700407	2,46 (++)
Enterococcus faecalis ATCC 29212	2,07 (++)
Escherichia coli O157:H7 IAL1848	2,93 (++)
Antibiotic susceptibility profile	I[2]
Resistance genes	Not detected
Growth at 43 °C	+
Growth at 48 °C	-
Hydrophobicity (%)	14,96
Self-aggregation (%)	84,03

[1]: Antagonistic activity. Halo/colony ratio = mean halo/colony ratio by species of *Lactococcus* spp., followed by the lowest and highest values in the range (in parentheses). *Halo/colony ratio < 1 = nonantagonistic (-); halo/colony ratio > 1 = regular antagonistic activity (+); halo/colony ratio > 2 = good antagonistic activity (++); halo/colony ratio > 3 = excellent antagonistic activity (+++).

[2]: I = Susceptible to all antibiotics tested; II = Susceptible to 8/9 antibiotics tested; III = Susceptible to 7/9 antibiotics tested; IV = Susceptible to 6/9 antibiotics tested; V = Susceptible to 5/9 antibiotics tested; VI = Susceptible to 4/9 antibiotics tested; VII = Susceptible to 3/9 antibiotics tested; VIII = Susceptible to 2/9 antibiotics tested; IX = Susceptible to 1/9 antibiotics tested; X = Susceptible to 0/9 antibiotics tested.

4. DISCUSSION

Considering the economic importance of bovine mastitis and the risk of antimicrobial resistance associated with this disease, the search for new prevention and treatment tools has intensified. One alternative to antibiotics is the use of LAB as mammary probiotics [19, 26, 27]. Commensal bacteria in the mammary gland, as LAB, along with minor mastitis-causing pathogens, such as coagulase-negative staphylococci, contribute to mammary homeostasis and play crucial roles in protecting the bovine host from colonization by major mastitis pathogens [13, 49]. These bacteria protect the host through several mechanisms, including competition with pathogens for nutrients, production of organic acids and antimicrobial compounds, inhibition of pathogen adhesion, and modulation of the mammary gland immune response [13].

Given rising antimicrobial resistance, probiotics, particularly immunomodulatory strains (immunobiotics), are emerging as promising approaches for managing and preventing bovine mastitis by enhancing host immunity and supporting a resilient mammary microbiome [29]. In this context, the prospection of autochthonous strains isolated from the mammary gland and/or raw milk is essential for building a portfolio of promising bacteria, as host specificity has been described as a favorable probiotic characteristic [50], and their effectiveness appears to be greater when these strains are obtained from their natural habitat [51].

Among the various genera and species of LAB, *L. lactis* shows promising potential as a probiotic, as certain strains and subspecies offer benefits and are recognized as globally safe (GRAS) [52]. Numerous studies have investigated the probiotic potential of *L. lactis* strains in humans, including their roles in regulating the gut microbiota with antidepressant [53], antihypertensive [54], and anticancer effects [55]. Similarly, in the control of bovine mastitis, *in vitro* studies have shown encouraging results [25, 26]. Furthermore, *L. lactis* has been tested as a treatment for this disease through intramammary infusions of live cultures [19, 27, 56, 57] and the antimicrobial peptide nisin [58 - 60]. However, it is important to note that some strains of *L. lactis* may have negative health effects on humans and animals, such as endocarditis in adults [61], diarrhea in children [62], and mastitis in dairy cows [28].

L. garvieae is considered the only pathogenic species of *Lactococcus* [63] and is known to cause septicemia, referred to as lactococcosis, which was first described in rainbow trout in Japan [64]. Since then, it has been recognized as a relevant pathogen in fish. In addition to being isolated in cases of bovine mastitis and dairy products [65], *L. garvieae* has also been identified as an emerging zoonotic pathogen [66]. On the other hand, this species is also a producer of bacteriocins known as garviecins [67]. Suneel and Basappa [68] identified and characterized a strain of *L. garvieae* isolated from raw milk that inhibited indicator pathogens such as *S. aureus* through the production of bacteriocin.

Therefore, it is crucial to differentiate potentially pathogenic strains of *Lactococcus* spp. isolated from infections from beneficial strains that have potential for application in the dairy production chain through phenotypic and genotypic characterization [52]. The screening of autochthonous strains to identify probiotic and bacteriocinogenic properties constitutes the first step in developing technologically advantageous cultures. These analyses are essential to ensure that the selected strains are effective in controlling pathogens and safe for intramammary application, without causing adverse effects in animals and ensuring the quality of milk intended for human consumption.

Armas et al. [25] investigated the *in vitro* antagonistic potential of the strain *L. lactis* LMG 7930 against mastitis-causing pathogens in ruminants, including *S. aureus, S. agalactiae*, *E. coli*, *Staphylococcus chromogenes*, *S. epidermidis*, *Staphylococcus intermedius*, and *Streptococcus dysgalactiae*. Similar to the findings of the present study, the authors reported that *L. lactis* inhibited most of the tested pathogens, with the highest antimicrobial activity observed against *S. agalactiae*. However, no inhibitory effect was observed for *E. coli*, which contrasts with the present research, where *L. lactis* strains exhibited a strong antagonistic effect against this pathogen, demonstrating that antimicrobial activity is a lineage-specific property [69]. Similar results to those of Armas et al. [25] were described by Akbar et al. [70], who noted greater antibacterial activity of *L. lactis* subsp. *lactis* strains isolated from fermented dairy products against *Listeria monocytogenes* and *S. aureus*, with less antagonistic effects against *Salmonella* Typhimurium and *E. coli*.

It is known that LAB can inhibit pathogenic microorganisms through various mechanisms, which involve the production of organic acids, such as lactic, acetic, and propionic acids, bacteriocins, hydrogen peroxide, and other antimicrobial metabolites [71]. In the present study, the ability of genetically distinct strains of *Lactococcus* spp. inhibit important bovine mastitis pathogens was evaluated using the spot-on-lawn technique. These antagonistic results allowed the screening of strains with antimicrobial potential for subsequent tests of probiotic properties.

Probiotic strains have the potential to control mastitis by antagonizing microorganisms and alleviating inflammation in the mammary gland. They can suppress intramammary pathogens through competitive exclusion or by producing antimicrobial compounds [13]. In the present study, no indications of bacteriocin production were observed in the evaluated *Lactococcus* spp. strains. These results suggest that the substances responsible for the antagonistic activity are likely not proteinaceous in nature, or do not essentially depend on the participation of protein molecules, considering the proteases used and the conditions tested. Further investigations should be conducted to elucidate the mechanisms involved in the antimicrobial activity of the strains against the tested indicator pathogens.

The safety of a probiotic strain is defined by the low pathogenic risk, and antibiotic resistance profile [72]. Susceptibility to antimicrobials is an important criterion for selecting probiotic strains because of the risks of lateral gene transfer of resistance markers to commensal and pathogenic members of the host's indigenous microbiota [73]. Antimicrobial resistance itself does not present significant implications for probiotic LAB isolates; however, this capacity to transfer resistance genes is the real cause of risk and must be investigated before commercial applications [74]. While LAB are intrinsically resistant to some antibiotics, food-isolated strains are typically susceptible to major clinical antibiotics such as ampicillin, penicillin, and gentamicin [73].

Based on the results of the antagonism assay, *Lactococcus* spp. strains (n = 24) that exhibited good to excellent antagonistic activity against at least 4 of the 6 tested indicator pathogens were selected to verify their antibiotic susceptibility. All evaluated *Lactococcus* strains were susceptible to ampicillin and chloramphenicol. Most strains were susceptible or moderately susceptible to penicillin, erythromycin, vancomycin, tetracycline, and gentamicin, which are commonly used antibiotics in intramammary infusions for the treatment of mastitis. On the other hand, most strains were resistant to streptomycin and cotrimoxazole. In line with the present work, Alharbi and Alsaloom [75] studied LAB isolated from raw milk and their antimicrobial activity against bacteria causing bovine mastitis. The authors also observed susceptibility to chloramphenicol in all tested strains and to ampicillin in most evaluated LAB, using the disk diffusion method. However, in contrast to the findings of this study, high resistance to tetracycline was observed. Supporting both the results of the present study and those of Alharbi and Alsaloom [75], Kondrotiene et al. [76] also reported a high occurrence of resistance to streptomycin and tetracycline in *L. lactis* strains isolated from raw and fermented milk, using the minimum inhibitory concentration (MIC) method. These results collectively emphasize the variability in resistance profiles among LAB, potentially influenced by strain origin and local antibiotic practices, as the extensive use of these antimicrobials in dairy herds.

From a One Health perspective, potentially probiotic LAB strains should be screened for antibiotic resistance genes, regardless of their phenotypic resistance [77]. Probiotic strains are recognized as safe if they have only a minimal possibility of transferring antibiotic resistance genes [78]. Among the resistance genes investigated in this study, only the genes *blaZ* and *tet*S were detected. The *blaZ* gene, a marker for beta-lactam resistance, was detected in only one strain: *L. lactis* CV252, which was moderately resistant to penicillin G and susceptible to ampicillin. Although *blaZ* is one of the most frequently detected beta-lactam resistance genes in *Staphylococcus* spp. [79], it is rarely detected in LAB [80, 81]. The *tet*S gene was identified in five strains that also exhibited resistance to tetracycline in the disk diffusion test.

Another important aspect in the prospecting of potentially probiotic LAB strains is their tolerance to elevated temperatures, which can be particularly relevant in controlling bovine mastitis. This thermal resistance is a desirable attribute that not only helps in distinguishing pathogenic isolates from beneficial strains [14], but also enhances the resilience of probiotics under stress conditions, such as those found in inflamed mammary glands. During mastitis, the mammary gland experiences increased temperatures due to the inflammatory response, which can inhibit the growth of some bacterial strains. Therefore, selecting LAB strains that can withstand these elevated temperatures is essential for their effective colonization and activity within the udder environment. In this study, the growth capacity of *Lactococcus* spp. strains at different temperatures was assessed as a key selection criterion for identifying superior strains. By demonstrating thermal tolerance, these strains are more likely to persist and exert their probiotic effects even under the adverse conditions associated with mastitis, potentially aiding in the reduction of pathogenic bacteria and supporting udder health.

On the basis of its antagonistic capacity against bovine mastitis pathogens and safety profile, the *L. lactis* CV151 was selected as the most suitable strain for the purposes of this study and proceeded to tests for cell surface characteristics. This strain demonstrated good antagonistic activity against all tested indicator pathogens and was susceptible to all the evaluated antimicrobials. Notably, no resistance genes were detected in its genome, which reinforces its safety for application.

The adhesion capacity of bacterial cells to hydrocarbons was assessed as an indicator of the hydrophobicity of the *L. lactis* CV151 strain [47]. Bacterial adhesion to hydrocarbons is widely used to determine the hydrophobicity of the cell surface in LAB, which is related to adhesion to epithelial cells [82]. Bacteria with greater hydrophobicity tend to bind more efficiently to epithelial cells. Furthermore, cell surface hydrophobicity plays a crucial role in facilitating initial contact between bacteria and host cells, influencing the overall adhesion capacity [83]. In this study, *L. lactis* CV151 exhibited low hydrophobicity, which may impact its effectiveness in colonizing epithelial surfaces.

The bacterial self-aggregation phenotype may indicate the ability to form a protective biofilm on the host mucosa [48], allowing for the maintenance of bacterial cells in the target epithelial tissue. Self-aggregation is an important indicator of the beneficial properties of potentially probiotic LAB [84]. Additionally, the high self-aggregative capacity of *L. lactis* CV151 suggests significant potential for its use in mastitis control strategies, contributing to herd health and milk quality. These results indicate that the strain possesses desirable characteristics that can be explored in future research and practices in the field of veterinary microbiology.

5. CONCLUSION

This study highlights the potential of indigenous *Lactococcus* spp., particularly *L. lactis* CV151, as probiotic candidates for bovine mastitis control. The demonstrated antagonistic activity against mastitis-causing pathogens, combined with a favorable antibiotic susceptibility profile, underscores their suitability for practical application. The findings emphasize the importance of further investigation into the probiotic properties of these strains to optimize their efficacy and safety. By advancing research in this area, this study lays a foundation for the development of sustainable and effective probiotic-based interventions, representing a promising alternative to conventional antimicrobial therapies in dairy cattle management.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions - CRediT

Joice Fátima Moreira Silva: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Bruna Vieira Alonso:** Investigation, Formal analysis, Conceptualization. **Paula Aparecida Azevedo Almeida:** Investigation, Formal analysis, Conceptualization. **Isabela Vieira Barbosa:** Investigation, Formal analysis. **Geraldo Márcio da Costa:** Conceptualization, Review & editing. **João Batista Ribeiro:** Supervision, Project administration, Funding acquisition, Conceptualization.

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CHAPTER - 4: Bioprotective potential of *Enterococcus* spp. strains isolated from raw milk against bovine mastitis pathogens

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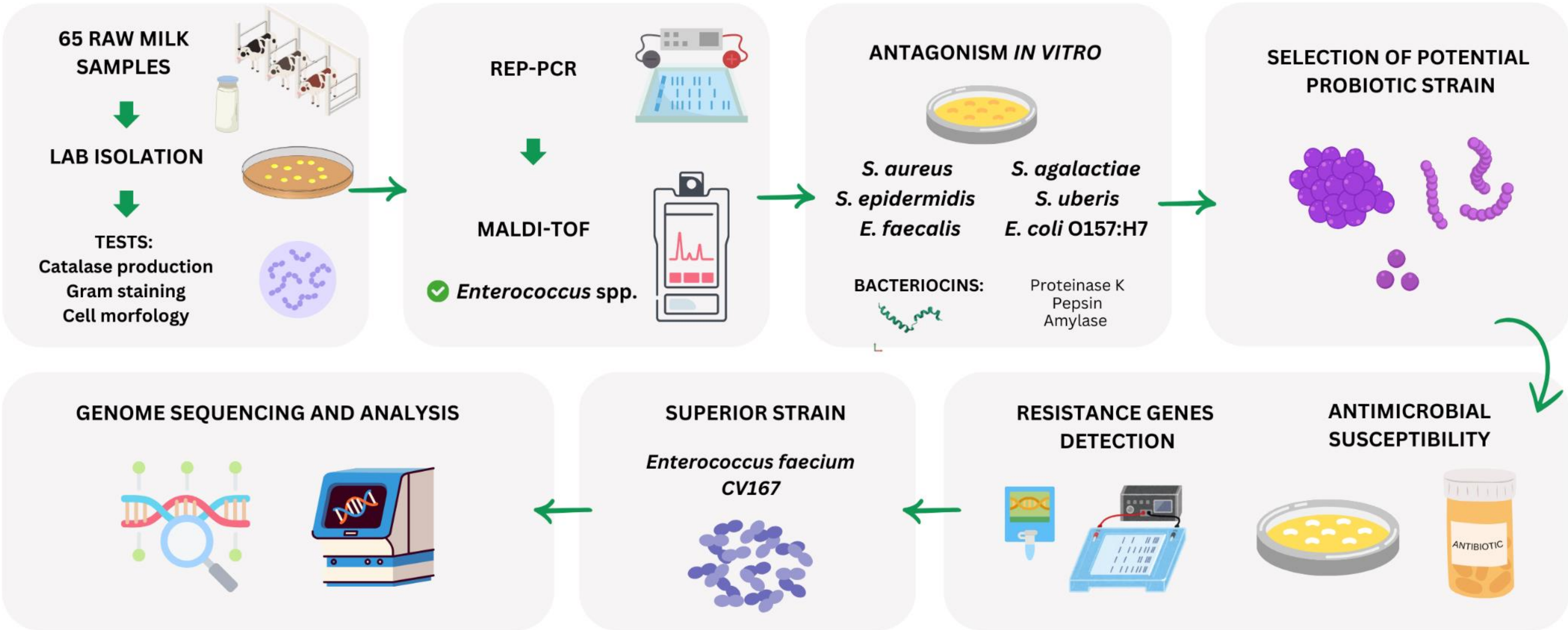
ABSTRACT

Mastitis is the most prevalent disease in dairy cows, causing substantial economic losses to producers and the dairy industry due to reduced milk production and quality. Antibiotics are commonly used for the prevention and treatment of this disease, but their widespread use contributes to antimicrobial resistance and environmental contamination. This study investigated the bioprotective potential of autochthonous *Enterococcus* spp. strains isolated from raw milk as a sustainable alternative to antibiotics. A total of 701 microorganisms were isolated from 65 raw milk samples, 368 of which were classified as lactic acid bacteria (LAB). A total of 221 LAB strains were identified as genetically distinct through rep-PCR and further classified by MALDI-TOF. Forty-two *Enterococcus* strains were selected for subsequent analyses. The antagonistic activity against six bovine mastitis-causing pathogens was evaluated, revealing that *Enterococcus* spp. strains exhibited good antagonistic activity against *S. aureus*, *S. agalactiae*, and *E. coli* O157:H7. Moreover, the *Enterococcus* spp. strains CV17 and CV167 showed evidence of bacteriocin production against *E. faecalis*. Four strains, including CV17 and CV167, were selected for further analysis based on their antagonistic activity and growth at 43 °C. These strains were susceptible to most antibiotics, with the exception of cotrimoxazole. One antibiotic resistance gene (*aacA-aphD*) was detected in *E. faecalis* CV373. Owing to the species' historical of safe use, *E. faecium* CV167 was selected as the most promising strain, and its genome was sequenced. Genomic analysis revealed the presence of six antibiotic resistance genes, one virulence gene and the presence of three bacteriocin-related genomic regions (Enterocin A, Enterocin B, and Enterolysin A). These findings highlight the promising antimicrobial potential of *E. faecium* CV167 and underscore the need for a comprehensive risk assessment before advancing this strain for probiotic applications in the control of bovine mastitis.

Keywords: antagonism, antimicrobial susceptibility, bacteriocins, genomic analysis.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Bovine mastitis is an inflammatory response in mammary tissue caused by physical trauma or infectious microorganisms [1]. It is considered the most common disease in dairy cows, leading to significant economic losses in the dairy industry due to reduced milk production and quality [2]. On average, the total cost associated with bovine mastitis is estimated at USD 147 per cow annually, resulting in an 11% to 18% decrease in the gross margin per cow per year [3]. Approximately 70% of these losses are attributed to reduced milk production due to tissue damage in the mammary gland [4].

Various risk factors, including pathogens, the host's immune system, and environmental factors, are associated with the incidence of bovine mastitis and play a significant role in disease occurrence [1]. To control mastitis, the use of antibiotics is a widely adopted practice, making the disease responsible for most antibiotic doses administered on dairy farms [5]. This approach is used both to treat active infections and to prevent new infections during the dry period. However, extensive antibiotic use contributes to the development of antimicrobial resistance, posing a significant challenge to animal and public health [6]. Moreover, the presence of antibiotic residues in milk can impact food safety and limit the marketability of dairy products [1]. Therefore, the search for effective and sustainable alternatives to antibiotic use is essential for mastitis control and the sustainability of the dairy production chain.

Bacteriocins are small, ribosomally synthesized antimicrobial peptides produced by both Gram-positive and Gram-negative bacteria [7, 8]. They have been described as being effective primarily against closely related bacterial species [9]. However, some studies emphasize their diverse spectrum and mechanisms of action [10], which enable antimicrobial activity against a wide variety of microorganisms, including bacteria, fungi, parasites, viruses, and even naturally resistant structures such as bacterial biofilms [8]. Bacteriocins form a highly heterogeneous family, varying in thermal stability, molecular weight, release and action modes, microbial targets, and protective mechanisms for the producing strains [7, 11]. Although described as being universally produced, most bacteriocins identified thus far originate from Gram-positive bacteria, such as lactic acid bacteria (LAB).

Bacteriocins derived from LAB have been widely applied across various fields, including the cosmetics industry [12], the food industry [13], and human and veterinary medicine [10, 14]. In animal production, bacteriocin-producing bacteria are employed as probiotics in the diets of swine, poultry, and fish, resulting in improved performance and productivity [15, 16]. Additionally, bacteriocins can be used in ruminant nutrition as alternatives to ionophores, mitigating the risks associated with antibiotic use in feeds [17].

In the control of bovine mastitis, bacteriocins present a promising alternative to antibiotics [17]. The use of these antimicrobial peptides for treating this disease has been explored since the early stages of scientific research into their applications [18]. Since then, bacteriocins with antimicrobial activity against mastitis-causing pathogens such as *Staphylococcus aureus*, *Escherichia coli*, and *Streptococcus* spp. have been identified, and commercial products for disease control are already available [14, 19, 20].

Enterococcus species are recognized as bacteriocin producers, in terms of both strain numbers and the diversity of enterocins they produce [21]. These antimicrobial peptides offer the added advantage of specifically inhibiting pathogens that cause bovine mastitis. Among these species, *Enterococcus faecium* stands out for its ability to produce bacteriocins that can inhibit the growth of genetically related or unrelated bacteria [22]. This diversity of action makes enterocins promising tools in the development of alternative therapeutic strategies for controlling infections in the mammary gland, potentially replacing or complementing the use of antibiotics in mastitis treatment.

Despite their extensive use for probiotic purposes [23], it is important to note that enterococci, especially *E. faecium* and *E. faecalis*, include strains that are multi-resistant and harbor genes encoding virulence factors. These bacteria are widely recognized for their pathogenic potential, generating significant concerns for global health [24]. Additionally, *Enterococcus* spp. are potential mammary gland pathogens and can cause mastitis [25-27]. In this context, enterocins themselves can be used against pathogenic *Enterococcus* strains [21]. Therefore, in the selection and use of strains from this genus for probiotic purposes, rigorous screening of effective and bacteriocinogenic strains is necessary, ensuring that their phenotypic and genotypic characteristics guarantee their safety for animal health applications.

The successful use of probiotic bacterial strains and their metabolites also requires the isolation of microorganisms from specific ecosystems, such as a particular herd or geographic region, followed by the selection and characterization of the bacteria and the antimicrobial mechanism responsible for their activity. Additionally, genome sequence analysis of promising probiotic *Enterococcus* strains is essential due to the potential impact of these bacteria on human and animal health. Whole-genome analysis serves as an ideal approach for detecting genomic variations, offering valuable insights into virulence, pathogenesis, antimicrobial resistance, host specificity, and phylogenetic relationships [28].

This study aimed to investigate the bioprotective potential of autochthonous *Enterococcus* spp. strains isolated from raw milk against bovine mastitis-causing pathogens. The objectives included the selection of bacteriocinogenic strains and, ultimately, the genomic analysis of the most promising strain to assess its probiotic properties, such as antimicrobial resistance markers and the biosynthesis of secondary metabolites and bacteriocins.

2. MATERIAL AND METHODS

2.1 Collection of raw milk samples

Raw milk samples were aseptically collected from the cooling tanks of 65 dairy herds across 14 municipalities in the Campo das Vertentes and Southern Minas Gerais regions, of Brazil. To preserve sample integrity during transport, 10% glycerol was added before freezing, and the samples were stored at -20 °C until analysis.

2.2 Isolation of lactic acid bacteria

The milk samples were thawed at room temperature for bacterial isolation. A 1 mL aliquot from each sample was serially diluted in phosphate-buffered solution (pH 6.2, Merck, Germany) from 10^{-1} to 10^{-3}. Then, 100 μL of each dilution were plated on Petri dishes containing MRS agar (BD Difco, USA) and M17 agar (Sigma-Aldrich, USA) culture media. The plates were subsequently incubated at 35 °C for 24 to 48 hours. Phenotypically distinct bacterial colonies were selected and streaked onto MRS or M17 agar for purification, according to the initial isolation medium, and incubated at 35 °C for 24 h. The isolates were then tested for catalase production, Gram-stained, and examined microscopically to determine their cellular morphology. All catalase-negative, Gram-positive cocci and rods were presumptively considered LAB. Finally, isolates were transferred to microtubes containing milk with 10% glycerol and stored at -20 °C and -80 °C for further characterization.

2.3 Molecular typing

To assess genetic similarity and exclude duplicates, LAB isolates were grouped using repetitive extragenic palindromic PCR (rep-PCR), according to Versalovic et al. [29]. Rep-PCR was performed with the universal primer (GTG)5 (5'-GTG GTG GTG GTG GTG-3'). Band patterns were analyzed using the Bionumerics software (version 6.6, Applied Maths, Sint-

Martens-Latem, Belgium) with the Jaccard coefficient. Cluster analyses of the combined rep-PCR data were performed using the unweighted pair group method with arithmetic means [30].

2.4 Identification by MALDI-TOF

Taxonomic identification of nonredundant LAB strains was conducted using matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry, following Nacef et al. [31]. Protein profiles were obtained from bacterial cultures using an ethanol/formic acid extraction protocol. Bacterial suspensions (200 µL) were washed twice with sterile Milli-Q water (16,000 × *g* for 1 min) and inactivated with ethanol (900 µL). Cellular proteins were then extracted with formic acid (10 µL at 70%) and pure acetonitrile (10 µL). After centrifugation (16,000 × *g* for 2 min), 1 µL of the supernatant from each sample was applied to a well on the MALDI plate (Bruker Daltonics) and air-dried at room temperature. Each sample was then coated with 1 µL of MALDI matrix solution (10 mg/mL of α-cyano-4-hydroxycinnamic acid, Sigma-Aldrich) in a solution containing 50% acetonitrile and 2.5% trifluoroacetic acid, and air-dried. Mass spectra were acquired on an Autoflex III SmartBeam mass spectrometer (Bruker Daltonics), and calibrated externally using the Bacterial Test Standard (Bruker Daltonics). Identification was deemed highly reliable for score values between 2.0 and 3.0, moderately reliable for values between 1.70 and 1.99, and values below 1.69 were considered nonidentifiable. An *Escherichia coli* strain was used as the standard control.

2.5 Selection criteria for LAB strains

For the purposes of this study, among the genetically distinct coccoid LAB strains identified by MALDI-TOF, *Enterococcus* spp. strains were selected for the evaluation of their antimicrobial and bacteriocinogenic activities against bovine mastitis pathogens.

2.6 Antagonistic activity of *Enterococcus* spp. against pathogens

The antagonistic activity of nonredundant *Enterococcus* spp. strains was evaluated using the spot-on-lawn method, as described by Tagg et al. [32] with modifications. Six bovine mastitis-causing pathogens were used as indicator bacteria: *Enterococcus faecalis* ATCC 29212, *E. coli* O157:H7 IAL1848, *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus uberis* ATCC 700407. LAB strains were cultured on MRS agar (35 °C for 24 h). After growth, three colonies from each LAB strain were transferred to 1,000 μL of MRS broth and incubated at 35 °C ± 2 °C for 24 h. Subsequently, 5 μL of each bacterial suspension was added to MRS agar in five equidistant spots. Plates were incubated aerobically at 35 °C ± 2 °C for 24 h. Separately, indicator bacteria were inoculated in 10 mL of BHI broth and incubated at 37 °C for 4 h. Then, 100 μL of each pathogen suspension culture was added to 7 mL of semi-solid BHI agar pre-warmed to 50 °C, and this mixture was poured over the MRS plate, forming a layer over the LAB spots. Petri dishes were then re-incubated aerobically for 14 to 18 h at 35 °C ± 2 °C. The presence of inhibition halos, measured in millimeters (mm) around the spots/colonies, indicated favorable antibacterial activity against the indicator pathogen [33]. The ratio between the size of the inhibition zone/halo (mm) and the size of the colony/spot (mm) was used in this study to determine antagonistic activity and classify strains according to their antagonistic effect, as the existing literature criteria are subjective and non-standardized. LAB strains with a halo/colony ratio below 1 were classified as non-antagonistic (-); those with a halo/colony ratio above 1 but below 2 were considered to have moderate antagonistic activity (+); those with a ratio above 2 but below 3 were classified as having good antagonistic activity (++); and LAB strains with a ratio above 3 were categorized as having excellent antagonistic activity (+++).

2.7 Assessment of the chemical nature of the inhibitory substances

The methodology proposed by Lewus & Montville [33] and adapted by dos Santos et al. [34] was used to evaluate the sensitivity of inhibitory substances produced by LAB to proteases. The same procedure described in the previous section was repeated with some modifications. Before the semisolid BHI was poured onto the Petri dish, 5 μL of an aqueous solution of proteinase K (20 mg/mL), amylase (20 mg/mL), and pepsin (20 mg/mL) were added beside the LAB spot/colony. The plates were incubated at 35 °C for two additional hours. Subsequently, 7 mL of semisolid BHI broth containing the indicator pathogen was poured over the same plates. The absence of an inhibition halo in the area where the protease was applied (half-moon shape) indicated the proteinaceous nature of the inhibitory substance. A control plate without added enzyme was used as a negative control, and the test was performed in triplicate.

2.8 Growth at different temperatures

To assess growth capacity at different temperatures, freshly isolated colonies from each selected *Enterococcus* spp. strain were inoculated in 1 mL of MRS broth and incubated at 35

°C for 12 h. Subsequently, 5 µL of the bacterial suspension were added to equidistant spots on Petri dishes containing MRS agar, in triplicate. The plates were incubated at temperatures of 35, 43, and 48 °C. After 48 h, the presence (+) or absence (-) of bacterial growth at the application sites was recorded.

2.9 Selection criteria for potentially probiotic *Enterococcus* spp. strains

Based on the results of antagonism assays, detection of proteinaceous inhibitory substances, and growth at different temperatures, *Enterococcus* spp. strains exhibiting good to excellent antagonistic activity against at least 4 of the 6 indicator pathogens tested, with or without evidence of bacteriocin production, and capable of growing at a minimum of 43 °C were selected. These strains were used for antimicrobial susceptibility characterization.

2.10 Evaluation of the antimicrobial susceptibility of *Enterococcus* spp.

Antimicrobial susceptibility testing was conducted using the disk diffusion method proposed by Bauer et al. [35], with modifications by dos Santos et al. [34]. The selected *Enterococcus* spp. strains were reactivated on MRS agar at 37 °C for 24 h to obtain isolated colonies. After growth and confirmation of isolate purity, three colonies were collected, inoculated into MRS broth, and incubated for 24 h at 37 °C. Subsequently, 100 µL of the culture were inoculated onto Petri dishes containing 25 mL of MRS agar and spread evenly across the plate using a Drigalski loop. Nine disks containing the following antimicrobials (Oxoid®, Basingstoke, UK) were then placed on the plate: penicillin G (10 µg), ampicillin (10 µg), vancomycin (30 µg), gentamicin (10 µg), streptomycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), erythromycin (15 µg), and cotrimoxazole (1.25 µg of trimethoprim and 23.75 µg of sulfamethoxazole). The plates were incubated aerobically for 24 h at 35 °C. To evaluate the results, inhibition zones (mm) were measured with a millimeter ruler, and strains were classified as susceptible or resistant to each antimicrobial according to Charteris et al. [36].

2.11 Detection of antibiotic resistance genes

The selected *Enterococcus* spp. strains were assessed for the presence of the following antibiotic resistance genes in their genomes: *erm*B, *tet*O, *tet*M, *tet*K, *tet*L, *tet*W, *tet*S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A, and *van*B. The detection of antibiotic resistance genes was performed via PCR following protocols previously described in the literature (Table 1).

Table 1. Primers used for detecting antibiotic resistance genes in selected LAB strains.

Target gene	Primer	Amplicon size (pb)	Reference
*erm*B	*erm*B 1 - CGTTTACGAAATTGG*AAC*AGGTAAAGGGC *erm*B 2 - GAATCGAGACTTGAGTGTGC	359	Lina et al. [37]
*tet*O	*tet*O 1 - *AAC*TTAGGCATTCTGGCTCAC *tet*O 2 - TCCCACTGTTCCATATCGTCA	515	Ng et al. [38]
*tet*M	*tet*M 1 - AGTTTTAGCTCATGTTGATG *tet*M 2 - TCCGACTATTTAGACGACGG	1862	Trzcinski et al. [39]
*tet*K	*tet*K 1 - TATTTTGGCTTTGTATTCTTTCAT *tet*K 2 - GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al. [39]
*tet*L	*tet*L 1 - ATAAATTGTTTCGGGTCGGTAAT *tet*L 2 - *AAC*CAGCC*AAC*TAATGACAATGAT	1077	Trzcinski et al. [39]
*tet*W	*tet*W 1 - GAGAGCCTGCTATATGCCAGC *tet*W 2 - GGGCGTATCCACAATGTT*AAC*	168	Aminov et al. [40]
*tet*S	*tet*S 1 - TGG*AAC*GCCAGAGAGGTATT *tet*S 2 - ACATAGACAAGCCGTTGACC	740	Obioha et al. [41]
*aac*A-*aph*D	*aac*A-*aph*D 1 - TAATCCAAGAGCAATAAGGGC *aac*A-*aph*D 2 - GCCACACTATCAT*AAC*CACTA	228	Strommenger et al. [42]
*mec*A	*mec*A 1 - TGGCTATCGTGTCACAATCG *mec*A 2 - CTGG*AAC*TTGTTGAGCAGAG	310	Vannuffel et al. [43]
*bla*Z	*bla*Z 1 - AAGAGATTTGCCTATGCTTC *bla*Z 2 - GCTTGACCACTTTTATCAGC	517	Vesterholm-Nielsen et al. [44]
*van*A	*van*A 1 - TCTGCAATAGAGATAGCCGC *van*A 2 - GGAGTAGCTATCCCAGCATT	377	Lemcke et al. [45]
*van*B	*van*B 1 - GCTCCGCAGCCTGCATGGACA *van*B 2 - ACGATGCCGCCATCCTCCTGC	529	Lemcke et al. [45]

2.12 Selection of the promising *Enterococcus* spp. strain

According to the bacteriocinogenic potential, antibiotic susceptibility profile and historical use of enterococci species, the strain that best suited the purpose of the study was finally selected as promising for whole genome sequencing (WGS).

2.13 Whole genome sequencing

For WGS of the *Enterococcus* spp. chosen as the promising strain, DNA was extracted using the phenol-chloroform method, according to Oliveira et al. [46]. The quantity and quality of the DNA were assessed via NanoDrop 1000 UV/Vis (Thermo Scientific, Massachusetts, EUA) and the sequencing library was prepared with the Illumina DNA Prep Kit. The DNA was then sequenced using 300-bp paired-end sequencing on the Illumina NextSeq 2000 platform. FastQC 0.12.1 [47] was initially used to assess the quality of the sequencing data. Paired-end reads were trimmed using Trimmomatic v.0.36, according to Bolger et al. [48], with the following parameters: trailing: 10, leading: 10, sliding window: 4:20, and a minimum length of 50 bp. *De novo* assembly of the short reads into contigs was performed using SPAdes 3.15.4 [49], with the coverage parameter set to "auto" and k-mers of 21, 33, 55, 77, 99, and 127. Contigs shorter than 500 bp were excluded from the final assembly. The assembly quality was evaluated using QUAST 5.2.0 [50]. Genome completeness was assessed using BUSCO 5.5.0 [51]. To confirm the taxonomic assignment, the assembled genome was analyzed using JSpecies [52] and genome annotation was performed using Prokka 1.14.16 [53].

2.13.1 Screening for virulence, mobile genetic elements, and antimicrobial resistance genes

Abricate software [54] was used to detect plasmids and identify antibiotic resistance and virulence genes. For this purpose, the databases Comprehensive Antibiotic Resistance Database (CARD) [55], ARG-ANNOT [56], Resfinder [57], National Center for Biotechnology Information (NCBI), and MEGARes [58] - for identifying antibiotic resistance genes; Virulence Factor Database (VFDB) [59] - for identifying virulence genes; and PlasmidFinder [60] - for identifying plasmids - were used. Additionally, the software Phastest [61] was employed to search for phage sequences within the genome of the sequenced strain. Coverage and identity cut-offs were set on the basis of the program’s default configurations (80% of minimum coverage and identity).

2.13.2 Identification of bacteriocin biosynthetic clusters

BAGEL4 [62] was used to search for bacteriocin biosynthetic clusters in the sequenced genome, and antiSMASH [63] was applied for the detection of biosynthetic clusters of secondary metabolites, with focus on bacteriocins.

3. RESULTS

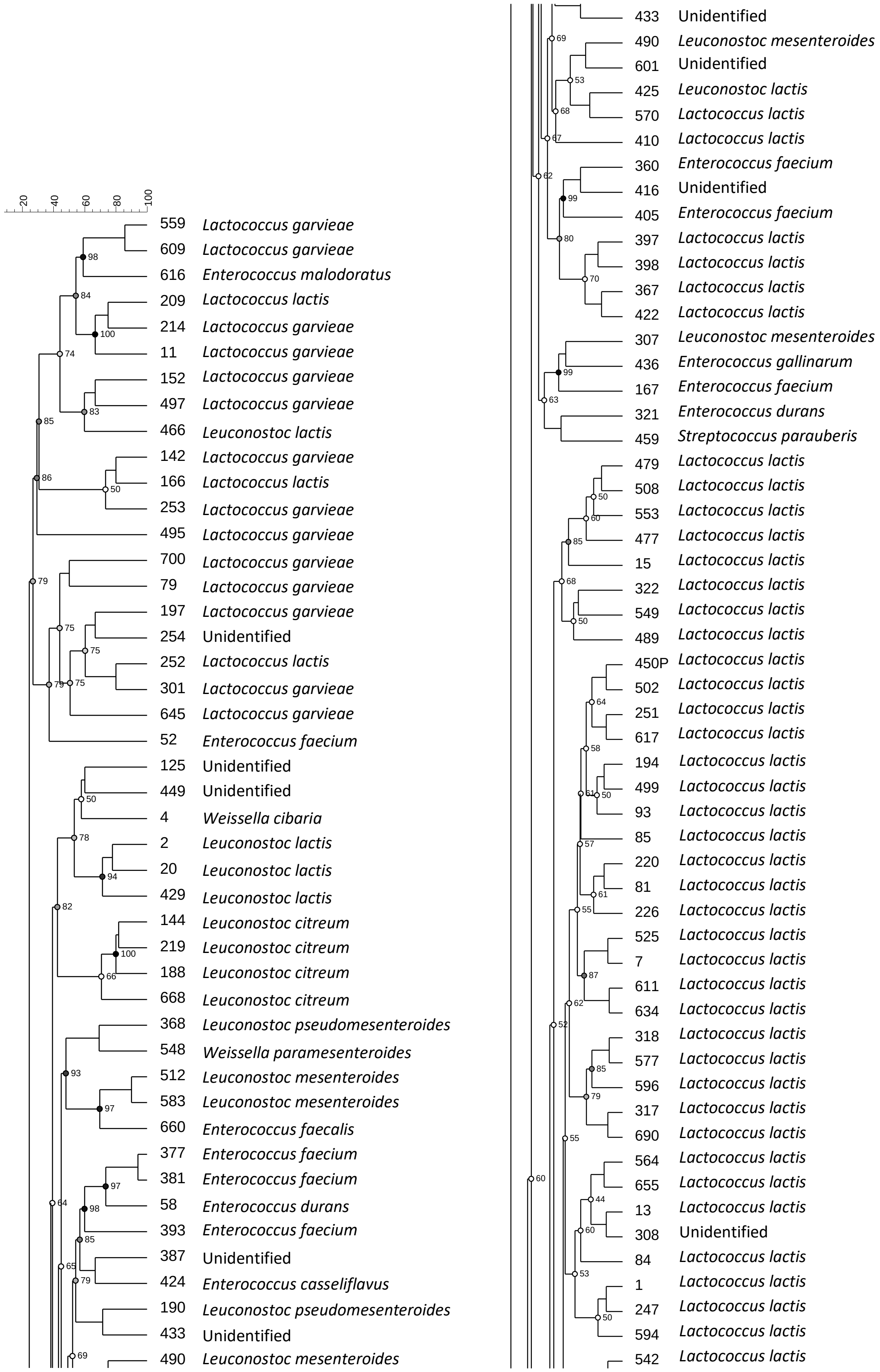
3.1 Isolation, genetic diversity, and identification of LAB

A total of 701 bacteria were isolated from raw milk samples collected from 65 dairy herds, 368 of which were Gram-positive and catalase-negative; therefore, these bacteria were presumptively classified as LAB. Among these strains, 210 were isolated on MRS culture medium, and 158 on M17. Morphologically, 94 isolates were identified as cocci, 220 as coccobacilli, and 54 as rods.

In the genetic similarity analysis using rep-PCR, the dendrogram (Figure 1) indicated that 221 isolates were genetically distinct and proceeded to MALDI-TOF identification. The MALDI-TOF analysis identified the following species: *Lactococcus lactis* (*n* = 129), *L. garvieae* (*n* = 18), *Enterococcus faecalis* (*n* = 23), *E. faecium* (*n* = 10), *E. durans* (*n* = 3), *E.*

casseliflavus (*n* = 2), *E. italicus* (*n* = 2), *E. gallinarum* (*n* = 1), *E. malodoratus* (*n* = 1), *Leuconostoc lactis* (*n* = 6), *L. citreum* (*n* = 5), *L. mesenteroides* (*n* = 7), *L. pseudomesenteroides* (*n* = 2), *Streptococcus parauberis* (*n* = 4), *Weissella cibaria* (*n* = 1), and *W. paramesenteroides* (*n* = 1). MALDI-TOF identification scores ranged from 1.7 to 2.41, indicating medium to high confidence levels.

In accordance with the selection criteria of this study, 42 genetically distinct *Enterococcus* spp. strains were selected for characterization of their antagonistic and bacteriocinogenic potential against mastitis-causing pathogens. The selected LAB strains belong to the following species: *E. faecalis* (*n* = 23), *E. faecium* (*n* = 10), *E. durans* (*n* = 3), *E. casseliflavus* (*n* = 2), *E. italicus* (*n* = 2), *E. gallinarum* (*n* = 1), and *E. malodoratus* (*n* = 1) (Table 2). The genetic diversity of the isolates, confirmed by rep-PCR, enabled the development of a collection of *Enterococcus* species and strains with distinct profiles and potential applications for the purpose of this study.


20
40
60
80
100
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616 Enterococcus malodoratus
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11 Lactococcus garvieae
152 Lactococcus garvieae
497 Lactococcus garvieae
466 Leuconostoc lactis
142 Lactococcus garvieae
166 Lactococcus lactis
253 Lactococcus garvieae
495 Lactococcus garvieae
700 Lactococcus garvieae
79 Lactococcus garvieae
197 Lactococcus garvieae
254 Unidentified
252 Lactococcus lactis
301 Lactococcus garvieae
645 Lactococcus garvieae
52 Enterococcus faecium
125 Unidentified
449 Unidentified
4 Weissella cibaria
2 Leuconostoc lactis
20 Leuconostoc lactis
429 Leuconostoc lactis
144 Leuconostoc citreum
219 Leuconostoc citreum
188 Leuconostoc citreum
668 Leuconostoc citreum
368 Leuconostoc pseudomesenteroides
548 Weissella paramesenteroides
512 Leuconostoc mesenteroides
583 Leuconostoc mesenteroides
660 Enterococcus faecalis
377 Enterococcus faecium
381 Enterococcus faecium
58 Enterococcus durans
393 Enterococcus faecium
387 Unidentified
424 Enterococcus casseliflavus
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433 Unidentified
490 Leuconostoc mesenteroides
601 Unidentified
425 Leuconostoc lactis
570 Lactococcus lactis
410 Lactococcus lactis
360 Enterococcus faecium
416 Unidentified
405 Enterococcus faecium
397 Lactococcus lactis
398 Lactococcus lactis
367 Lactococcus lactis
422 Lactococcus lactis
307 Leuconostoc mesenteroides
436 Enterococcus gallinarum
167 Enterococcus faecium
321 Enterococcus durans
459 Streptococcus parauberis
479 Lactococcus lactis
508 Lactococcus lactis
553 Lactococcus lactis
477 Lactococcus lactis
15 Lactococcus lactis
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489 Lactococcus lactis
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634 Lactococcus lactis
318 Lactococcus lactis
577 Lactococcus lactis
596 Lactococcus lactis
317 Lactococcus lactis
690 Lactococcus lactis
564 Lactococcus lactis
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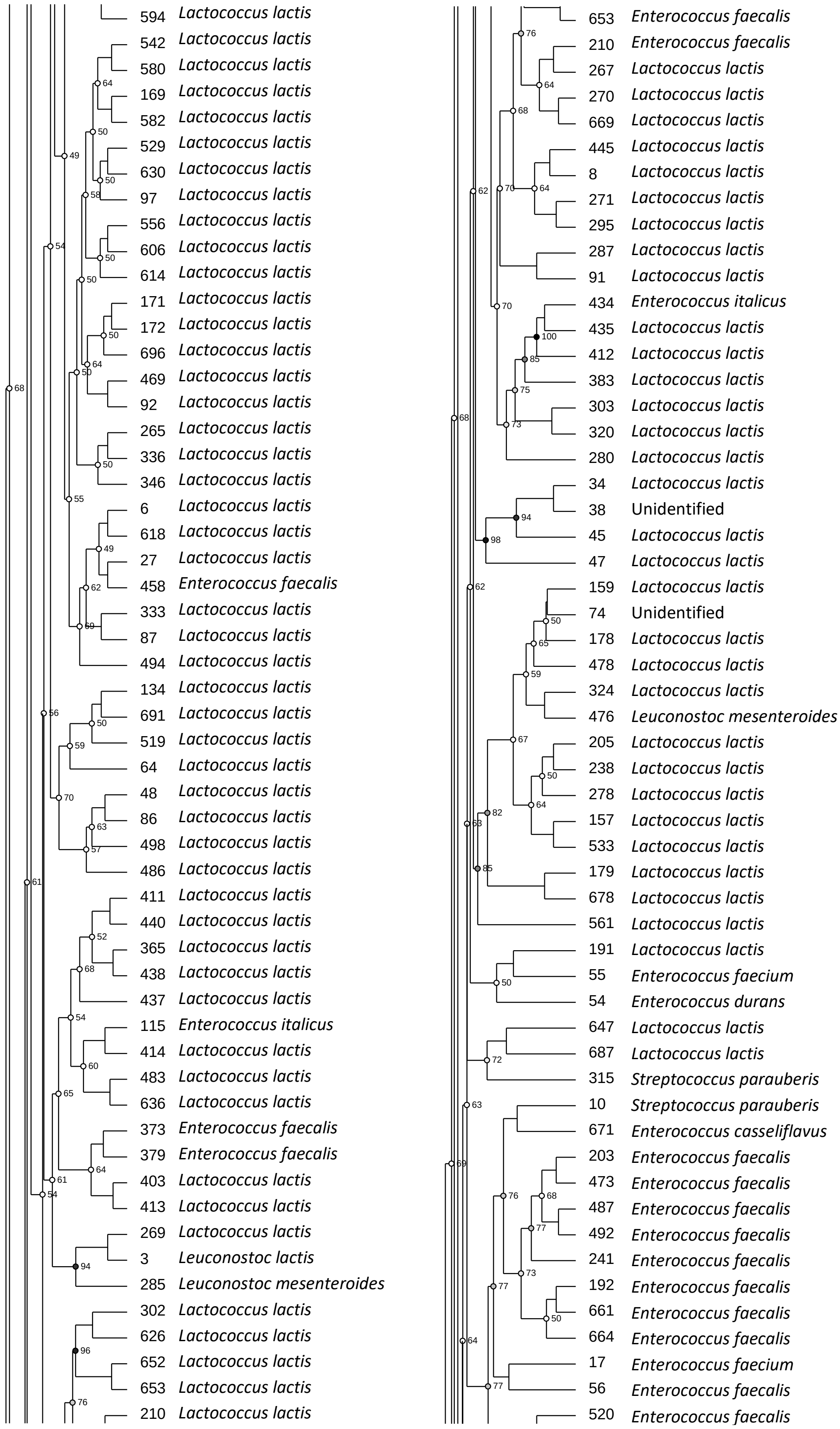
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542 Lactococcus lactis
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134 Lactococcus lactis
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519 Lactococcus lactis
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86 Lactococcus lactis
498 Lactococcus lactis
486 Lactococcus lactis
411 Lactococcus lactis
440 Lactococcus lactis
365 Lactococcus lactis
438 Lactococcus lactis
437 Lactococcus lactis
115 Enterococcus italicus
414 Lactococcus lactis
483 Lactococcus lactis
636 Lactococcus lactis
373 Enterococcus faecalis
379 Enterococcus faecalis
403 Lactococcus lactis
413 Lactococcus lactis
269 Lactococcus lactis
3 Leuconostoc lactis
285 Leuconostoc mesenteroides
302 Lactococcus lactis
626 Lactococcus lactis
652 Lactococcus lactis
653 Lactococcus lactis
210 Lactococcus lactis
653 Enterococcus faecalis
210 Enterococcus faecalis
267 Lactococcus lactis
270 Lactococcus lactis
669 Lactococcus lactis
445 Lactococcus lactis
8 Lactococcus lactis
271 Lactococcus lactis
295 Lactococcus lactis
287 Lactococcus lactis
91 Lactococcus lactis
434 Enterococcus italicus
435 Lactococcus lactis
412 Lactococcus lactis
383 Lactococcus lactis
303 Lactococcus lactis
320 Lactococcus lactis
280 Lactococcus lactis
34 Lactococcus lactis
38 Unidentified
45 Lactococcus lactis
47 Lactococcus lactis
159 Lactococcus lactis
74 Unidentified
178 Lactococcus lactis
478 Lactococcus lactis
324 Lactococcus lactis
476 Leuconostoc mesenteroides
205 Lactococcus lactis
238 Lactococcus lactis
278 Lactococcus lactis
157 Lactococcus lactis
533 Lactococcus lactis
179 Lactococcus lactis
678 Lactococcus lactis
561 Lactococcus lactis
191 Lactococcus lactis
55 Enterococcus faecium
54 Enterococcus durans
647 Lactococcus lactis
687 Lactococcus lactis
315 Streptococcus parauberis
10 Streptococcus parauberis
671 Enterococcus casseliflavus
203 Enterococcus faecalis
473 Enterococcus faecalis
487 Enterococcus faecalis
492 Enterococcus faecalis
241 Enterococcus faecalis
192 Enterococcus faecalis
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664 Enterococcus faecalis
17 Enterococcus faecium
56 Enterococcus faecalis
520 Enterococcus faecalis

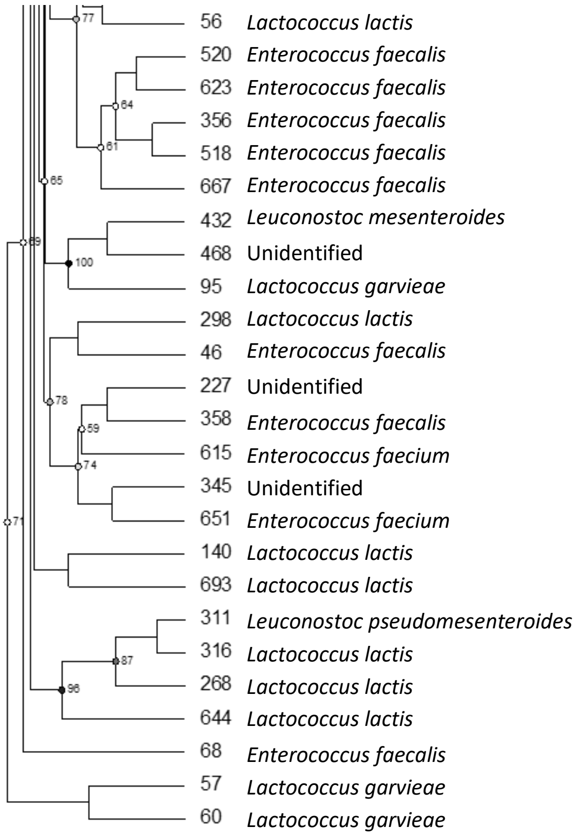

Figure 1. Dendrogram based on the cluster analysis of fingerprints by rep-PCR and identification of lactic acid bacteria (LAB) species using MALDI-TOF. Bionumerics software (version 6.6, Applied Maths, Sint-Martens-Latem, Belgium).

3.2 Antagonistic activity of *Enterococcus* spp. against pathogens

The antagonistic capacity of the genetically distinct *Enterococcus* spp. strains, determined by the spot-on-lawn technique (Figure 2 - A), is presented in Table 2. Overall, the *Enterococcus* spp. strains showed good antagonistic activity against the indicator pathogens *S. aureus* ATCC 25923, *S. agalactiae* ATCC 12386, and *E. coli* O157:H7 IAL1848, and moderate antagonistic activity against *S. epidermidis* ATCC 12228, *E. faecalis* ATCC 29212, and *S. uberis* ATCC 700407.

By species, *E. faecalis*, the most represented among the isolates (n = 23), demonstrated good antagonism against *S. aureus* and *E. coli*, and moderate antagonistic effects against *S. agalactiae*, *S. epidermidis*, *S. uberis*, and *E. faecalis*. In contrast, *E. faecium* strains (n = 10) exhibited good antagonistic activity against most indicator pathogens (*S. aureus*, *S. agalactiae*, *S. uberis*, and *E. coli*). Among other *Enterococcus* species with fewer isolates, *E. durans* (n = 3) showed good antagonistic activity against *S. agalactiae*, *S. uberis*, and *E. coli*, and moderate antagonistic effects against *S. aureus*, *S. epidermidis*, and *E. faecalis*. On average, *E. casseliflavus* (*n* = 2) exhibited good antagonistic activity against *S. aureus*, *S. agalactiae*, and *E. coli*. Meanwhile, *E. italicus* (*n* = 2) displayed good antagonistic activity against most indicator pathogens (*S. aureus*, *S. agalactiae*, *S. uberis*, and *E. coli*), moderate antagonism against *S. epidermidis*, and no antagonism against *E. faecalis*. *E. gallinarum* (*n* = 1) demonstrated moderate antagonistic effects against *S. aureus*, *S. epidermidis*, *S. uberis*, and *E. coli*, with no antagonism against *S. agalactiae* and *E. faecalis*. Finally, *E. malodoratus* (*n* = 1) showed good antagonistic activity against *E. coli*, moderate antagonism against *S. aureus*, *S. agalactiae*, and *S. uberis*, and no antagonism against *S. epidermidis* and *E. faecalis*.

Individually, *E. faecalis* CV17 and *E. faecium* CV167 exhibited excellent antagonistic activity against *E. coli* O157:H7, with halo/colony ratios of 3.12 and 3.0, respectively. *E. faecium* CV167 also showed strong antagonism against most pathogens except *S. epidermidis*, *E. faecalis* CV46 demonstrated excellent antagonism against *S. aureus* (halo/colony ratio = 3.08), *E. faecalis* CV203 showed excellent antagonistic activity against *S. uberis* (halo/colony ratio = 3.22), and *E. faecalis* CV373 exhibited good antagonistic effects against 4 out of the 6 indicators.

In terms of the chemical nature of the inhibitory substances, *E. faecalis* CV17 and *E. faecium* CV167 potentially produce bacteriocins, as indicated by reduced inhibition halos at the application points of the enzymes proteinase K, pepsin, and amylase (Fig. 1 – B, C, and D, respectively) against the pathogen *E. faecalis* ATCC 29212.

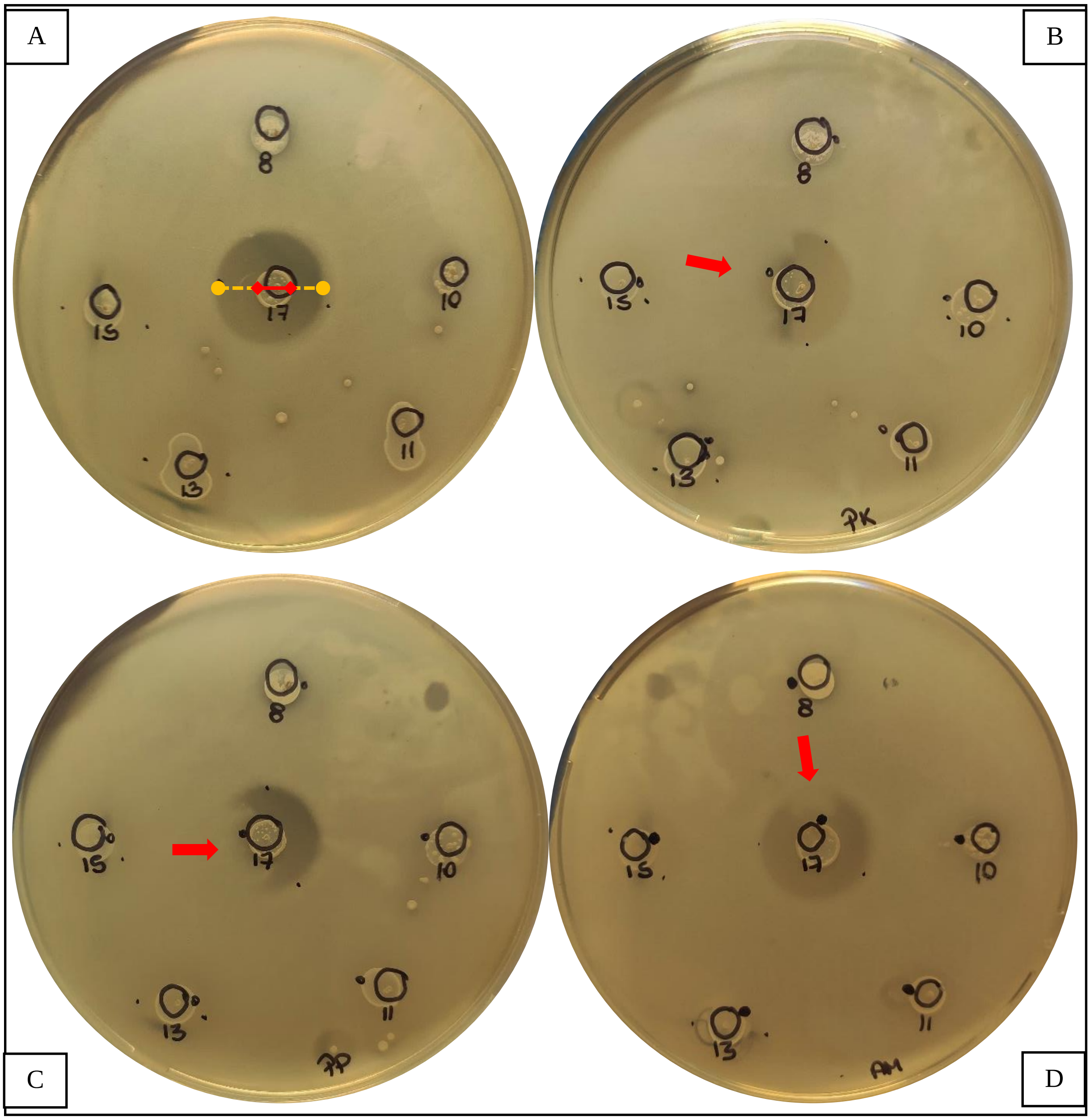

Figure 2. Antagonistic activity of *Enterococcus faecalis* CV17 against the pathogen *Enterococcus faecalis* ATCC 29212 via spot-on-lawn technique and detection of the chemical nature of the inhibitory substance. The halo/colony ratio (yellow dashed line for halo / solid red line for colony) represents the antagonistic activity of the *Enterococcus faecalis* strain CV17 against *E. faecalis* ATCC 29212 (**A**). The red arrows in the figures indicate the reduction in inhibition halos at the application points of the enzymes proteinase K (**B**), pepsin (**C**), and amylase (**D**).

3.3 Growth at different temperatures

Regarding the growth of *Enterococcus* spp. at various temperatures after 48 hours of incubation, all strains grew at 35 °C (isolation temperature), 21 strains (50%) grew at 43 °C, and 11 strains (26.2%) grew at 48 °C.

3.4 Antimicrobial susceptibility of *Enterococcus spp.* and detection of resistance genes

Following the selection criteria of this study, four strains of *Enterococcus spp.* were chosen for antimicrobial susceptibility testing based on their antagonistic activity against pathogens, indication of bacteriocinogenic activity, and ability to grow at relatively high temperatures. These strains included *E. faecalis* CV17, CV46, and CV373, and *E. faecium* CV167.

Data on the antimicrobial susceptibility, resistance profile, and detection of antibiotic resistance genes for the selected strains are presented in Table 3. All four selected strains of *Enterococcus* spp. were susceptible to ampicillin, chloramphenicol, and vancomycin and were resistant to cotrimoxazole. *E. faecalis* CV46 was resistant to only one antibiotic tested (cotrimoxazole), classifying it under susceptibility profile II (susceptible to 8 of 9 antibiotics tested). *E. faecalis* CV17 was susceptible to 6 of the 9 antibiotics tested, displaying susceptibility profile IV. *E. faecium* CV167 and *E. faecalis* CV373 were susceptible to three antibiotics: ampicillin, chloramphenicol, and vancomycin, falling under susceptibility profile VII (susceptible to 3 of the 9 antibiotics tested).

With respect to the detection of antibiotic resistance genes, only the *E. faecalis* CV373 strain carried a resistance gene, *aacA-aphD*. Consequently, this strain was excluded from further analyses because of the potential risk of transferring resistance markers to other beneficial or pathogenic bacteria.

Table 2. MALDI-TOF identification and antagonistic activity of *Enterococcus* spp. strains isolated from raw milk against bovine mastitis-causing pathogens**:** *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *Staphylococcus epidermidis* ATCC 12228, *Streptococcus uberis* ATCC 700407, *Enterococcus faecalis* ATCC 29212, and *Escherichia coli* O157:H7 IAL1848.

MALDI-TOF identification		**Indicator pathogens Mean halo/colony ratio followed by the range of values** [2]					
LAB species	***n***[1]	***S. aureus***	***S. agalactiae***	***S. epidermidis***	***S. uberis***	***E. faecalis***	***E. coli***
Enterococcus faecalis	23	2.28 [1.43 - 3.08] (++)*	1.98 [0.0 - 2.79] (+)	1.43 [0.0 - 2.07] (+)	1.84 [0.0 - 3.22] (+)	1.61 [0.0 - 2.95] (+)	2.26 [1.51 - 3.12] (++)
Enterococcus faecium	10	2.03 [0.0 - 2.62] (++)	2.29 [1,29 - 2.73] (++)	1.60 [1.28 - 1.95] (+)	2.13 [1.77 - 2.61] (++)	1.56 [0.0 - 2.00] (+)	2.47 [1.32 - 3.00] (++)
Enterococcus durans	3	1.95 [1.83 - 2.03] (+)	2.48 [2.42 - 2.51] (++)	1.45 [1.37 - 1.58] (+)	2.33 [2.00 - 2.96] (++)	1.70 [1.63 - 1.78] (+)	2.15 [2.03 - 2.28] (++)
Enterococcus casseliflavus	2	2.01 [1.78 - 2.23] (++)	2.07 [1.84 - 2.30] (++)	1.39 [1.16 - 1.62] (+)	1.77 [1.28 - 2.26] (+)	1.33 [1.30 - 1.35] (+)	2.38 [1.88 - 2.87] (++)
Enterococcus italicus	2	2.38 [2.21 - 2.55] (++)	2.56 [2.44 - 2.68] (++)	1.60 [1.58 - 1.62] (+)	2.45 [2.23 - 2.66] (++)	0.79 [0.0 - 1.58] (-)	2.46 [2.07 - 2.85] (++)
Enterococcus gallinarum	1	1.55 (+)	0.0 (-)	1,24 (+)	1.44 (+)	0.0 (-)	1.77 (+)
Enterococcus malodoratus	1	1.58 (+)	1.24 (+)	0.0 (-)	1.00 (+)	0.0 (-)	2.29 (++)
Total of strains	42	2.15 [0.0 - 3.08] (++)	2.06 [0.0 - 2.79] (++)	1.44 [0.0 - 2.07] (+)	1.94 [0.0 - 3.22] (+)	1.49 [0.0 - 2.95] (+)	2.30 [1.32 - 3.12] (++)

[1]: Number of LAB strains isolated per species.

[2]: Halo/colony ratio = mean halo/colony ratio by species of *Enterococcus* spp., followed by the lowest and highest values in the range (in parentheses).

[*]Halo/colony ratio < 1 = nonantagonistic (-); halo/colony ratio > 1 = regular antagonistic activity (+); halo/colony ratio > 2 = good antagonistic activity (++); halo/colony ratio > 3 = excellent antagonistic activity (+++).

Table 3. Susceptibility of *Enterococcus* spp. strains to the antibiotics ampicillin, penicillin, chloramphenicol, co-trimoxazole, erythromycin, streptomycin, gentamicin, tetracycline, and vancomycin by the disk diffusion method, susceptibility profile, and resistance genes detection.

***Enterococcus* spp. strains selected**	***Enterococcus* spp. strains susceptible to the antibiotics tested[1]**									**Susceptibility profile[2]**	**Resistance genes detected[3]**
	AMP	**PEN**	**CHL**	**SXT**	**ERI**	**STR**	**GEN**	**TET**	**VAN**		
Enterococcus faecalis CV17	S	S	S	R	S	R	S	R	S	IV	-
Enterococcus faecalis CV46	S	M	S	R	S	S	S	S	S	II	-
Enterococcus faecium CV167	S	R	S	R	R	R	R	R	S	VII	-
Enterococcus faecalis CV373	S	R	S	R	R	R	R	R	S	VII	*aacA-aphD*

S = susceptible; MS = moderately susceptible; R = resistant.

[1]: AMP = Ampicillin; PEN = Penicillin; CHL = Chloramphenicol; SXT = Co-trimoxazole; ERI = Erythromycin; STR = Streptomycin; GEN = Gentamicin; TET = Tetracycline; VAN = Vancomycin. [2]: I = Susceptible to all antibiotics tested; II = Susceptible to 8/9 antibiotics tested; III = Susceptible to 7/9 antibiotics tested; IV = Susceptible to 6/9 antibiotics tested; V = Susceptible to 5/9 antibiotics tested; VI = Susceptible to 4/9 antibiotics tested; VII = Susceptible to 3/9 antibiotics tested; VIII = Susceptible to 2/9 antibiotics tested; IX = Susceptible to 1/9 antibiotics tested; X = Susceptible to 0/9 antibiotics tested.

[3]: Resistance genes tested: *ermB, tetO, tetM, tetK, tetL, tetW, tetS, blaZ, mecA, aacA-aphD, vanA, and vanB*

Table 4. Probiotic and bacteriocinogenic properties of selected *Enterococcus spp.* strains isolated from raw milk with potential for bovine mastitis control.

Probiotic characteristics	***Enterococcus faecalis* CV17**	***Enterococcus faecium* CV167**
Staphylococcus aureus ATCC 25923	2.40[1] (++)*	2.29 (++)
Streptococcus agalactiae ATCC 12386	2.11 (++)	2.31 (++)
Staphylococcus epidermidis ATCC 12228	1.70 (+)	1.84 (+)
Streptococcus uberis ATCC 700407	1.80 (+)	2.06 (++)
Enterococcus faecalis ATCC 29212	2.95 (++)	2.00 (++)
Escherichia coli O157:H7 IAL1848	3.12 (+++)	3.00 (+++)
Potential bacteriocin producer?	Yes	Yes
Inhibitory substance sensitive to enzyme(s):	Proteinase K, Pepsin, and Amylase	Pepsin and Amylase
Growth at 43 °C	+	+
Growth at 48 °C	+	+
Antibiotic susceptibility profile	IV[2]	VII
Resistance genes	Not detected	Not detected

[1]: Antagonistic activity: Halo/colony ratio = mean halo/colony ratio by species of *Enterococcus* spp., followed by the lowest and highest values in the range (in parentheses).
[*]Halo/colony ratio < 1 = nonantagonistic (-); halo/colony ratio > 1 = regular antagonistic activity (+); halo/colony ratio > 2 = good antagonistic activity (++); halo/colony ratio > 3 = excellent antagonistic activity (+++).
[2]: I = Susceptible to all antibiotics tested; II = Susceptible to 8/9 antibiotics tested; III = Susceptible to 7/9 antibiotics tested; IV = Susceptible to 6/9 antibiotics tested; V = Susceptible to 5/9 antibiotics tested; VI = Susceptible to 4/9 antibiotics tested; VII = Susceptible to 3/9 antibiotics tested; VIII = Susceptible to 2/9 antibiotics tested; IX = Susceptible to 1/9 antibiotics tested; X = Susceptible to 0/9 antibiotics tested.

3.5 Genome sequencing, assembly and annotation

Based on the results obtained from the antagonistic activity against mastitis-causing pathogens (bacteriocinogenic potential), antibiotic susceptibility profile and historically established safe use of the species, the *E. faecium* CV167 strain was considered promising for the purpose of the study. Consequently, it was selected for complete genome sequencing to conduct in-depth analyses regarding its safety and potential for antimicrobial metabolite production.

The sequencing data generated 11,863,824 reads, of which only 1.37% were removed after trimming. *De novo* assembly generated 61 contigs with a total length of 2,736,418 bp, a GC content of 37.93%, and an N50 of 156,530 (Figure 3). The genome completeness was 99.3% for the Bacilli class. Taxonomic analysis revealed 99.45% nucleotide identity with *Enterococcus faecium* SRR24 (GenBank accession: GCA_009734005.2), with a tetra score of 0.99745. Genome annotation identified 2,622 coding sequences, 7 rRNAs, 62 tRNAs, and 1 tmRNA.

This Whole Genome Shotgun project has been deposited in the European Nucleotide Archive (ENA) under the accession number PRJEB79846. The raw Illumina reads are available under accession number ERR13648363 and the assembled genome is available under accession number ERZ24834527.

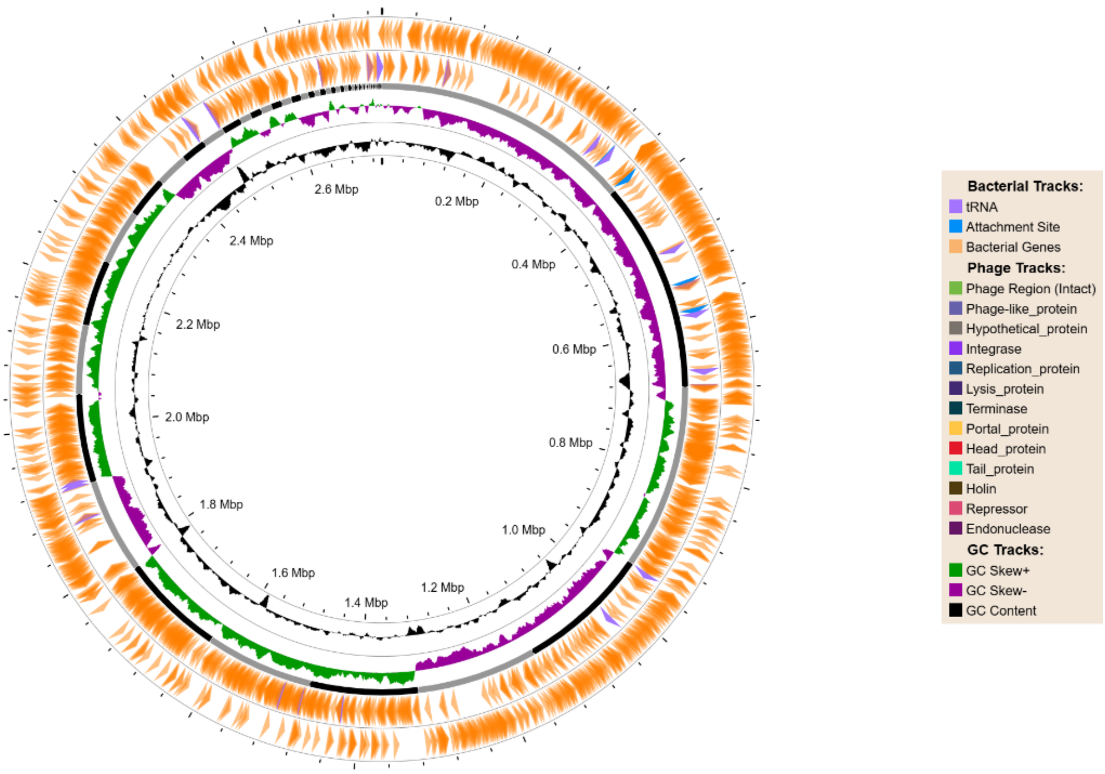

Figure 3. Genomic structure of *Enterococcus faecium* CV167. PlasmidFinder software.

3.6 Detection of plasmids, phage and genes of virulence and antimicrobial resistance in the genome

Analysis of the sequenced genome of the *E. faecium* CV167 strain revealed the presence of four replicons (Table 5). The presence of two phage sequences was also observed using the Phastest software (Table 6).

The results of resistance gene detection in the genome of the *E. faecium* CV167 strain are detailed in Table 7. Six resistance genes were identified: *aac*(6')-*Ii*, *tet*(L), *tet*(M), *msr*(C), *eat*(A), and *efm*A. Of these, *aac*(6')-*Ii*, *tet*(L), *tet*(M), and *msr*(C) were detected by all five databases used in the analysis—Argannot, CARD, ResFinder, NCBI, and MEGARes. The *efmA* gene was identified by the CARD and MEGARes databases, whereas *eat(A)* was uniquely identified through the NCBI database. Each gene displayed a coverage of 100%, and the minimum sequence identity was above 94% across all genes, underscoring the high reliability of the results.

The presence of virulence-related genes in the *E. faecium* CV167 strain was also investigated. Among the various *Enterococcus* virulence genes available in the VFDB database, only the *acm* gene, which encodes the collagen adhesin precursor, was identified (Table 6).

3.7 Identification of bacteriocin biosynthetic clusters

The analysis of biosynthetic clusters for bacteriocins in *E. faecium* CV167 revealed the presence of several clusters encoding enterocin variants. Using the BAGEL4 software, three genomic regions associated with bacteriocin production were identified, specifically encoding for enterocin A, enterocin B, and enterolysin A (Figure 4.A-C). Additionally, antiSMASH analysis of secondary metabolite biosynthetic clusters confirmed the presence of a biosynthetic cluster encoding enterocin A (Figure 5).

Table 5. Plasmid replicons detected in the *Enterococcus faecium* CV167 genome.

Plasmid Gene/replicon	% Coverage	% Identity	Plasmid reference
repUS15_2_repA(pNB2354p1)	100	100	CP004064
rep1_1_repE(pAMbeta)	95.04	96.82	AF007787
rep22_1b_repB(pAMalpha1)	88.46	100	AF503772
rep2_1_orf1(pRE25)	100	100	X92945

Table 6. Phage sequences detected in the *Enterococcus faecium* CV167 genome.

Phage	Region Length	Total Proteins	Region Position
PHAGE_Entero_vB_IME196_NC_028990(4)	40.3Kb	64	335552-375913
PHAGE_Entero_vB_IME197_NC_028671(8)	45.9Kb	66	161357-207351

Table 7. Resistance and virulence genes detected in the *Enterococcus faecium* CV167 genome.

Gene detected	Database	% Coverage	% Identity	Resistance/Virulence mechanism	Drug class resistance/ Virulence
aac(6')-Ii	Argannot	100	99.82	Antibiotic inactivation -aminoglycoside N-acetyltransferase AAC(6')-Ii	Aminoglycoside
	Card	100	99.82		
	Resfinder	100	99.82		
	NCBI	100	99.82		
	Megares	100	99.82		
***tet*(L)**	Argannot	100	100	Antibiotic efflux - tetracycline efflux MFS transporter Tet(L)	Tetracycline
	Card	100	99.78		
	Megares	100	100		
	Resfinder	100	100		
	NCBI	100	100		
***tet*(M)**	Argannot	100	95.62	Antibiotic target protection - tetracycline resistance ribosomal protection protein Tet(M)	Tetracycline
	Card	100	95.21		
	Megares	100	99.95		
	Resfinder	100	96.46		
	NCBI	100	99.95		
***msr*(C)**	Argannot	100	98.92	Antibiotic target protection - ABC-F type ribosomal protection protein MsrC	Streptogramin, streptogramin B, macrolide
	Card	100	94.39		
	Megares	100	98.92		
	Resfinder	100	98.92		
	Ncbi	100	98.92		
***eat*(A)**	NCBI	100	100	ABC-F type ribosomal protection protein Eat(A)	Pleuromutilin
efmA	Card	100	100	Antibiotic efflux pump	Fluoroquinolone, macrolide
	Megares	100	100		
acm	VFDB	97.97	99.86	Interacts with collagen type I and to a lesser extent with collagen type IV.	Collagen adhesin precursor

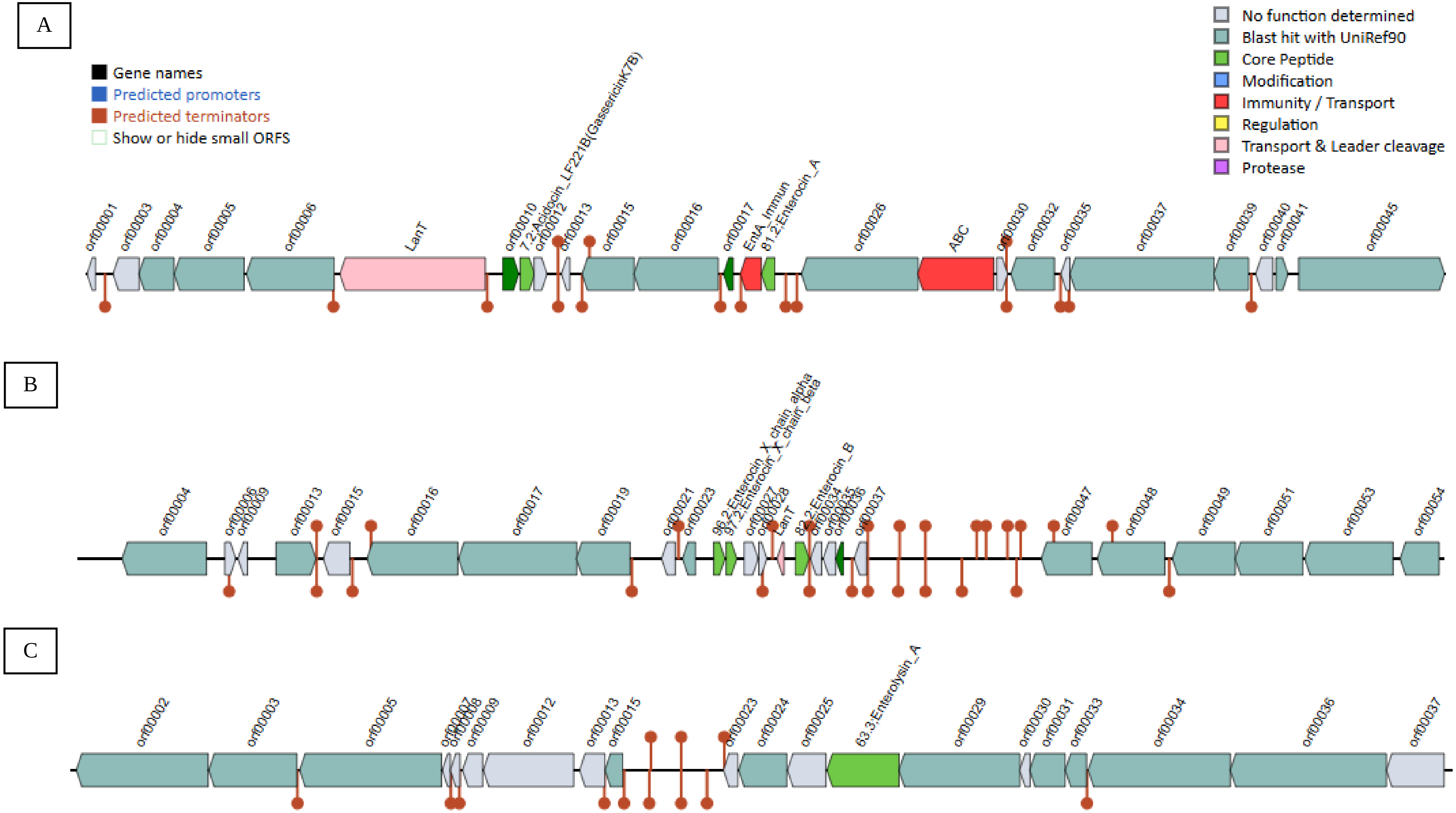

Figure 4. Bacteriocin gene clusters identified from *Enterococcus faecium* CV167 using BAGEL 4. **(A)** Enterocin A; **(B)** Enterocin B; **(C)** Enterolysin A.

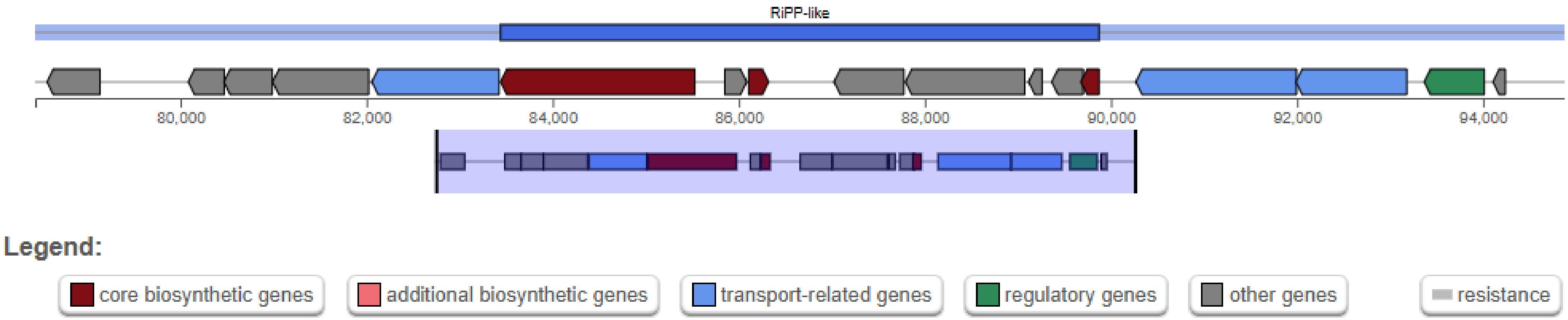

Figure 5. Bacteriocin gene clusters identified from *Enterococcus faecium* CV167 via antiSMASH.

4. DISCUSSION

In this study, from a large collection of microorganisms isolated directly from the raw milk of Brazilian dairy herds, *Enterococcus* strains from different species were selected to assess their antimicrobial potential against major pathogens responsible for bovine mastitis. *Enterococcus* spp. are important producers of bacteriocins (enterocins) [64], which are desirable antimicrobial agents due to their safety and low risk of residues in food and the environment. However, some *Enterococcus* species, particularly *E. faecalis* and *E. faecium*, are nosocomial pathogens responsible for bacteremia, endocarditis, urinary tract infections, intra-abdominal and pelvic infections, and even death in immunocompromised individuals [65]. This duality arises from the potential presence of genes encoding toxins, virulence factors, and antimicrobial resistance [66]. Therefore, screening *Enterococcus* isolates to study their biotechnological and probiotic potential, as well as their safety, is essential for selecting strains with bioprotective properties.

The genetic diversity of the LAB collection investigated in this study enabled the selection of *Enterococcus* spp. strains with strong antagonistic activity against key pathogens responsible for bovine mastitis, particularly *S. aureus*, *S. agalactiae*, and *E. coli*. These findings hold significant implications in the context of mastitis, given that the majority of cases are attributed to infections caused by staphylococci, streptococci, or coliform bacteria [67]. The ability of these selected *Enterococcus* strains to inhibit multiple mastitis-related pathogens suggests their potential as a natural, complementary approach for mastitis control.

The antimicrobial properties of LAB are mediated by the antimicrobial molecules they produce, which can be grouped into three main categories: bacteriocins, organic acids (acetic, butyric, and lactic acids), and other small molecules, such as diacetyl, hydrogen peroxide, acetaldehyde, acetoin, reuterin, and reutericyclin [68]. In the present study, the *E. faecalis* CV17 and *E. faecium* CV167 strains demonstrated potential bacteriocin production using the spot-on-lawn technique against the pathogen *E. faecalis* ATCC 29212, as shown by the inhibition halos that retracted in areas treated with proteases. These results suggest that the molecules responsible for the antagonistic activity of both strains against the indicator pathogen are likely proteinaceous in nature, given the loss of antimicrobial activity following protease treatment. Notably, bacteriocins generally exhibit high antimicrobial activity against closely related species [64], which explains the bacteriocinogenic effects of both strains, *E. faecalis* CV17 and *E. faecium* CV167, against the pathogenic *E. faecalis* ATCC 29212. This potential bacteriocinogenic effect served as a criterion for selecting promising strains for bioprotection purposes.

Tolerance to elevated temperatures is considered a desirable probiotic attribute, helping to differentiate pathogenic isolates from beneficial strains [20]. In this current study, the growth capacity of *Enterococcus* spp. strains at elevated temperature was also used as a criterion to select promising candidates. Beyond identifying potentially beneficial strains, resilience to adverse conditions of the mammary gland, including temperature increases due to inflammation, is essential for assessing the probiotic efficacy and practical applicability of these strains.

Despite the established use of *Enterococcus* spp., particularly *E. faecium,* as probiotics in human and animal health [66], the application of strains from these bacteria requires careful investigation regarding antibiotic resistance, as resistant *E. faecium* strains have been detected in commercial probiotic products for animals [69]. In this study, all four *Enterococcus* spp. strains selected for their antagonistic activity and growth at elevated temperatures were susceptible to ampicillin, chloramphenicol, and vancomycin, but resistant to co-trimoxazole (trimethoprim-sulfamethoxazole). These findings align with those of Boranbayeva et al. [70], who investigated the susceptibility of LAB strains isolated from traditional fermented dairy products and the feces of Holstein calves and heifers. The authors also reported that most cocci-shaped LAB strains were resistant to cotrimoxazole. Enterococcal strains can exhibit intrinsic resistance to various antibiotics, including cephalosporins, cotrimoxazole, lincomycin, and low levels of penicillin and aminoglycosides [71]. Furthermore, resistance of enterococci isolated from animal-derived foods to other antibiotics, such as erythromycin and tetracycline, has also been described as a common characteristic [72], as was indeed observed in the present study.

One of the most important factors in evaluating enterococci for use as starter cultures or probiotics is their resistance to glycopeptides, such as vancomycin [73], which serves as a key indicator in assessing the safety of LAB. However, several reports indicate that vancomycin resistance in lactic acid bacteria can be intrinsic, highlighting the need for more specific studies to draw stronger conclusions about safety concerning sensitivity to this antibiotic [74]. In the present study, none of the selected strains presented resistance to vancomycin, and no related resistance genes were detected.

The absence of acquired and transferable antibiotic resistance genes is one of the required properties by which specific strains can be considered potential probiotic bacteria [74, 75]. Among the four selected *Enterococcus* spp. strains, only *E. faecalis* CV373 harbored a resistance gene, identified as *aacA-aphD*, which encodes aminoglycoside resistance. This gene encodes a bifunctional enzyme that confers cross-resistance to clinically important aminoglycosides, including gentamicin, tobramycin, kanamycin, and, when overexpressed, amikacin [42]. Indeed, *E. faecalis* CV373 was resistant to gentamicin. This result underscores the importance of thorough antibiotic resistance screening in potential probiotic strains. Ensuring that probiotic candidates are free of such resistance markers is essential for their safe application in both human and veterinary health.

In Brazil, the National Health Surveillance Agency (ANVISA), through Resolution RDC No. 241 of July 26, 2018 [76], stipulates that probiotics must be susceptible to at least two clinically relevant antibiotics to ensure that effective treatment options are available in cases of infection [77]. The antimicrobial susceptibility results from this study suggest that, according to the ANVISA criteria, three strains evaluated (*E. faecalis* CV17 and CV46, and *E. faecium* CV167) may be considered for future research and potentially used as probiotics. Nevertheless, for the purposes of this present study, the *E. faecium* CV167 strain was chosen as a promising candidate because the species' established safe use as a probiotic in both pharmaceutical formulations and animal nutrition [78, 79]. Furthermore, this strain showed evidence of bacteriocin production against the pathogen *E. faecalis* ATCC 29212. Accordingly, its genome was sequenced to allow a deep and detailed assessment of its safety and bacteriocinogenic potential.

Genome sequencing confirmed the identification of strain CV167 as *E. faecium.* Assembly of the genome resulted in complete circular chromosome contigs with a total length of 2,736,418 bp, which is consistent with the genome sizes reported for other *E. faecium* strains [79, 80]. This genomic confirmation reinforces the classification of CV167 within the *E. faecium* species, providing a foundation for genetic and functional analyses relevant to its potential probiotic properties.

E. faecium CV167 presented four replicons. Mobile genetic elements, including plasmids and transposons, play a crucial role in the horizontal transfer of resistance determinants in enterococci [81]. Additionally, plasmids can ensure their own replication and inheritance [82]. Rosvoll et al. [82] investigated a large collection of *E. faecium* isolates from various sources and reported a high prevalence of plasmids, with between 1 and 7 plasmids detected in 88 out of 93 isolates. The authors reported that the strains with the greatest number of plasmids were also the most pathogenic. Further analyses of the replicons identified in CV167 will be conducted to clarify the implications of these plasmids for the potential use of the strain as a probiotic.

E. faecium is known to harbor bacteriophages [79, 82], and indeed, two phage sequences were identified in the CV167 strain. Bacteriophages play a crucial role in bacterial evolution, as they are capable of integrating into and excising from the bacterial genome, thereby facilitating horizontal gene transfer [84]. Under certain conditions, these phages can confer new genetic traits, such as antibiotic resistance, adaptation to specific environments, and increased virulence, to the host bacterium [85]. This mechanism contributes to the genetic diversity and adaptability of bacterial strains, which may have important implications for the safety and functionality of strains used as probiotics.

Genomic analysis also revealed that *E. faecium* CV167 carries several antibiotic resistance genes, including determinants for tetracycline resistance [*tet*(L) and *tet*(M)], macrolide resistance [*msr*(C) and *efmA*], aminoglycoside resistance [*aac(6')-Ii*], and pleuromutilin resistance [*eat*(A)]. The presence of these genes indicates a complex resistance profile, which could have implications for its safety and potential use as a probiotic. Additional analyses will be conducted to determine whether these genes are located in mobile genetic elements, which would represent a high risk of cross-resistance if horizontally transferred to pathogenic or commensal strains.

Notably, the *tet*(L) gene, which encodes efflux proteins, and the *tet*(M) gene, which is responsible for encoding ribosomal protection proteins, are commonly found in *E. faecium* strains isolated from food and environmental sources [80, 81]. Despite targeting these specific tetracycline resistance genes by PCR, they were not detected in strain CV167, likely due to primer inefficiency or limitations in PCR sensitivity. However, phenotypic testing via disk diffusion confirmed that *E. faecium* CV167 is indeed resistant to tetracycline. This discrepancy highlights the importance of genomic analysis in investigating the resistance mechanisms responsible for the phenotypic resistance observed in CV167. Similarly, although phenotypically resistant to erythromycin, the presence of the *erm*(B) gene, which encodes erythromycin resistance, was not detected by PCR. Nevertheless, genomic analysis revealed two other genes that may confer macrolide resistance: *msr*(C) and *efm*A. Ghattargi et al. [79] conducted a comparative genomic analysis of both probiotic and pathogenic *E. faecium* strains, reporting the presence of a macrolide-efflux transmembrane protein responsible for drug efflux, a mechanism that plays a crucial role in drug resistance in certain probiotic strains. The presence of these efflux pump mechanisms can explain the results observed in the present study.

In terms of virulence, genes associated with adherence in *Enterococcus* spp. are typically linked to pathogenic strains. In this study, only the *acm* gene, which encodes the collagen adhesin precursor, was identified. Ghattargi et al. [79] also detected this gene in a probiotic *E. faecium* strain, noting a mutation that suggested that it may be nonfunctional. Further investigations are needed to determine the functionality of the *acm* gene in *E. faecium* CV167.

On the other hand, for a probiotic agent to be effective, it must successfully colonize the target environment, which requires efficient adhesion mechanisms [88]. Furthermore, the pathogenicity of a bacterium is not solely dependent on its ability to adhere. While adhesion is essential for initial colonization and is often the first step for a pathogen to establish infection, additional virulence factors are required for disease progression [1]. In the case of *E. faecium* CV167, the presence of adhesins should not necessarily disqualify it as a probiotic candidate. In fact, this feature may be beneficial, as adhesive capability could enable *E. faecium* CV167 to establish and maintain itself within the mammary gland, creating a protective barrier that prevents colonization by more virulent pathogens and promotes host health. Thus, the presence of adhesins may represent a strategic advantage [89], facilitating both colonization and probiotic activity without implying pathogenicity.

The diversity and genomic plasticity of *E. faecium* are responsible for both probiotic and pathogenic nature [79]. Probiotic bacteria are particularly recognized for their ability to synthesize a variety of bioactive compounds, which confer antimicrobial activity against competing microorganisms [80]. In this study, several genomic regions linked to the production of bacteriocins—specifically enterocin A, enterocin B, and enterolysin A—were identified using the BAGEL4 and antiSMASH software platforms. These findings highlight the diverse biosynthetic potential of the *E. faecium* CV167 genome, with multiple pathways contributing to bacteriocin synthesis. The identification of these gene clusters supports the results from antagonism tests, confirming that *E. faecium* CV167 possesses significant antimicrobial potential, which likely contributes to its probiotic properties.

The genomes of *E. faecium* may contain diverse gene clusters encoding bacteriocins, and evidence suggests that these bacteriocins are significantly associated with specific host environments, indicating potential adaptation to particular hosts [90]. Enterocin A, classified as a class IIa pediocin-like bacteriocin, has been identified in isolates from various sources and displays inhibitory activity against a broad range of Gram-positive bacteria, including *Staphylococcus* spp., *Listeria* spp., and multiple lactic acid bacteria [21]. Enterocin B (subclass IId) is strongly associated with animal and food sources, aligning with previous studies that report its low occurrence in human clinical samples [21, 88]. Enterolysin A is a large, heat-labile bacteriocin, characteristics typical of class III bacteriocins [91], that exhibits activity against several genera, including *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Enterococcus*, *Bacillus*, *Listeria*, and *Staphylococcus* spp. [92]. The larger structure of enterolysin A may enable it to interact with a several microbial targets, suggesting a unique functional advantage in certain environments. These different activity profiles highlight the potential application of *E. faecium* CV167 bacteriocins in the control of mastitis pathogens.

Enterocin genes typically exhibit low expression levels in wild strains [93]. In natural environments, this reduced expression may reflect energy-saving adaptations, with bacteriocin production occurring only under specific conditions, such as competition with other microbial species. To increase enterocin production for broader applications, future research could focus on the generation of recombinant proteins through heterologous enterocin expression, such as in *E. coli* or *Lactococcus lactis*. Therefore, the safety risks identified in the genomic analysis of the *E. faecium* CV167 strain could be mitigated by employing a heterologous model for bacteriocin purification. The use of a heterologous model for bacteriocin expression and purification could help mitigate the safety risks identified in the genomic analysis of *E. faecium* CV167, making it feasible to use purified bacteriocins or employ safer probiotic strains.

As a future perspective, an additional approach to mitigate the risk of transmitting resistance genes present in *E. faecium* CV167 is genome editing through the CRISPR-Cas system. This technique is particularly attractive for combating antimicrobial resistance because it can precisely target and eliminate specific resistance genes or selectively kill only the targeted bacteria by cleaving their genomes [94]. This method could be applied to *E. faecium* CV167 to remove unwanted resistance elements, enhancing its safety profile as a probiotic strain for potential applications in sensitive environments, such as the mammary gland. Future research focused on developing CRISPR-based strategies for targeting resistance genes in probiotic bacteria holds promise for advancing safer, more effective biotherapeutic agents in veterinary contexts.

5. CONCLUSION

In summary, complete genome sequencing of *E. faecium* CV167 revealed the absence of most virulence-associated genes and key antibiotic resistance determinants, such as vancomycin resistance genes. At the same time, several genomic regions associated with bacteriocin production, which are likely responsible for its probiotic potential, were identified. However, the significant presence of plasmids and additional resistance genes requires further investigation to ensure the safety of the CV167 strain. These findings underscore the necessity of a comprehensive risk assessment prior to advancing *E. faecium* CV167 for probiotic applications. Although the species has a history of safe use, the detection of genomic resistance determinants highlights the importance of cautious and controlled application, particularly in clinical or agricultural settings, where antibiotic use could promote horizontal gene transfer to other microorganisms.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions - CRediT

Joice Fátima Moreira Silva: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Yasmin Neves Vieira Sabino:** Formal analysis, Data curation. **Bruna Vieira Alonso:** Investigation, Formal analysis, Conceptualization. **Paula Aparecida Azevedo Almeida:** Investigation, Formal analysis, Conceptualization. **Isabela Vieira Barbosa:** Investigation, Formal analysis. **Geraldo Márcio da Costa:** Conceptualization, Review & editing. **João Batista Ribeiro:** Supervision, Project administration, Funding acquisition, Conceptualization.

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CHAPTER - 5: *Weissella cibaria* CV4: a powerful strain isolated from raw milk against bovine mastitis pathogens

Brief Report

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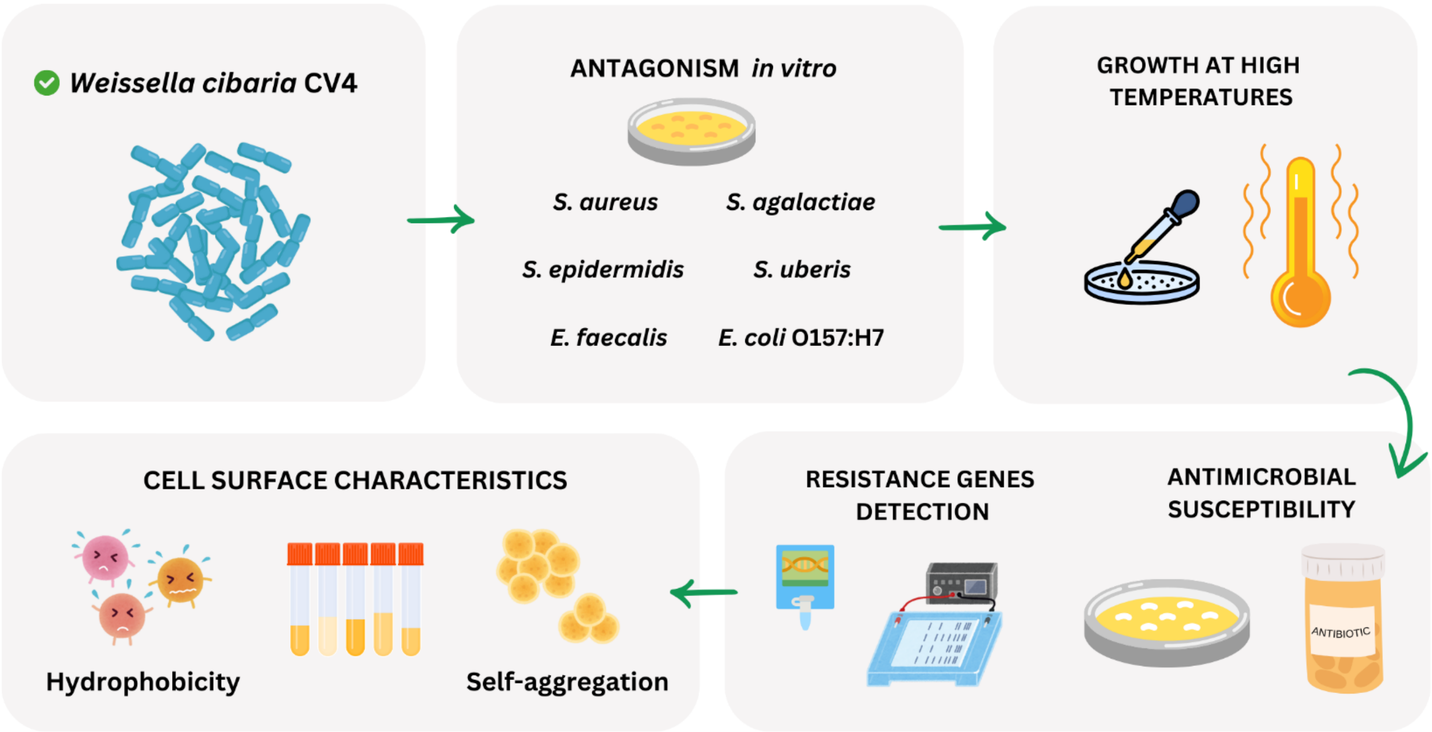
ABSTRACT

Bovine mastitis remains a major challenge in dairy production, especially due to the rising occurrence of antimicrobial resistance of its bacterial agents. This study reports the high *in vitro* antimicrobial activity of *Weissella cibaria* CV4, a strain isolated from raw milk, against six major bovine mastitis-causing pathogens. Using the spot-on-lawn assay, the strain demonstrated excellent antagonistic activity against *E. coli* O157:H7, *S. aureus*, *S. agalactiae*, and *S. epidermidis*. Additionally, *W. cibaria* CV4 demonstrated the ability to grow at elevated temperatures (43 and 48 °C), suggesting environmental resilience. The disc diffusion method revealed that the strain was susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline, but resistant to penicillin, cotrimoxazole, streptomycin, gentamicin, and vancomycin. Importantly, no antibiotic resistance genes (*erm*B, *tet*O/M/K/L/W/S, *bla*Z, *mec*A, *aac*A-*aph*D or *van*A/B) were detected through PCR analysis. *W. cibaria* CV4 also exhibited moderate hydrophobicity and a high self-aggregation rate (70.5%), characteristics that highlight its potential as a probiotic agent. These findings suggest that *W. cibaria* CV4 possesses a promising multifaceted profile, combining antimicrobial activity, thermal tolerance, and favorable adherence traits, making it a potential candidate for further development as a biocontrol agent for mastitis. Future research should explore the genetic mechanisms underlying its antimicrobial activity and resistance, ensuring its safety and effectiveness in mastitis control.

Keywords: antibiotic resistance, antimicrobial activity, lactic acid bacteria, probiotic.

GRAPHICAL ABSTRACT



1. INTRODUCTION

Bovine mastitis is one of the most prevalent and economically impactful diseases in the dairy production chain, caused primarily by pathogens such as *Staphylococcus aureus*, *Streptococcus* spp., and *Escherichia coli* (Berardo et al., 2022; Cheng & Han, 2020; Elias et al., 2020). Mastitis control has largely relied on antibiotic treatments, but the effectiveness of these interventions is increasingly compromised due to rising antimicrobial resistance among pathogens (Pérez et al., 2020). This phenomenon poses a threat not only to animal health but also to public health (Tomanić et al., 2023), as antimicrobial resistance genes may potentially be transferred between pathogens and commensal microorganisms (Brody et al., 2008).

In response to these challenges, alternatives to antibiotics are being explored, with particular interest in mammary probiotics for mammary gland use (Berardo et al., 2022; Kober et al., 2022; Rainard & Foucras, 2018). These beneficial microorganisms, when introduced directly into the mammary gland environment, can compete with pathogens, produce antimicrobial compounds that inhibit their growth, and modulate the host immune system, thereby promoting a safer and more sustainable approach for mastitis control (Kober et al., 2022). In this context, the species *Weissella cibaria* has garnered attention for its probiotic potential and antimicrobial properties (Yu et al., 2019).

This study reports the high *in vitro* antimicrobial activity of *Weissella cibaria* CV4, a lactic acid bacterium (LAB) strain isolated from raw milk, against six major bovine mastitis-causing pathogens. Characterizing this strain may provide valuable insights for the development of effective alternative treatments for intramammary infections, contributing to a reduction in antibiotic use in dairy farming.

2. MATERIAL AND METHODS

2.1 Origin, isolation and identification

The *W. cibaria* CV4 strain was isolated from raw milk collected from a dairy herd in Três Corações, MG, Brazil. For its isolation, a 1 mL aliquot of the milk sample was diluted in phosphate buffer (Merck, Germany) and serially diluted (10^{-1} to 10^{-3}) before plating on MRS agar (BD Difco, USA). Plates were incubated at 35 °C for 24 hours, and phenotypically distinct bacterial colonies were selected, streaked for purification, and incubated under the same conditions. Isolates were then tested for catalase production, Gram-stained, and examined microscopically to determine cell morphology. Catalase-negative, Gram-positive cocci or bacilli were classified as presumptive LAB.

To identify the isolates, genetic similarity was initially analyzed to exclude LAB clones using the repetitive extragenic palindromic PCR (rep-PCR) technique, according to Versalovic et al. (1991). Band patterns were analyzed with the Bionumerics software (v. 6.6, Applied Maths) using the Jaccard coefficient. Taxonomic identification of nonredundant LAB strains was performed by MALDI-TOF, following the procedure proposed by Nacef et al. (2017). Protein profiles were acquired in triplicate from bacterial cultures using ethanol/formic acid extraction. After a series of washes and ethanol inactivation, the bacterial sediment was treated with formic acid and acetonitrile for protein extraction. Mass spectra were obtained with an Autoflex III SmartBeam mass spectrometer (Bruker Daltonics) using external calibration and specific parameters for analysis. *E. coli* was used as a positive control. Strain CV4 was identified as *W. cibaria* with a score of 2.15, indicating a high-confidence identification.

2.2 Antagonistic activity of the *Weissella cibaria* CV4 strain against mastitis pathogens

The antagonistic activity of the *W. cibaria* CV4 strain was evaluated using the spot-on-lawn technique, as proposed by Tagg et al. (1976), with modifications. Six bovine mastitis-causing pathogens were used as indicator bacteria: *Staphylococcus aureus* ATCC 25923, *Streptococcus agalactiae* ATCC 12386, *Enterococcus faecalis* ATCC 29212, *Escherichia coli* O157:H7 IAL1848, *Staphylococcus epidermidis* ATCC 12228, and *Streptococcus uberis* ATCC 700407. LAB was cultured on MRS agar at 35 °C for 24 h. After growth, three colonies were incubated in MRS broth at 35 °C for 24 h. The bacterial suspension (5 μL) was applied to form spots on MRS agar plates in duplicate and incubated aerobically at 35 °C for 24 h. Meanwhile, the indicator bacteria were incubated in BHI broth (37 °C for 4 h), then mixed with semi-solid BHI agar (50 °C) and poured over the MRS plates to cover the LAB spots. Plates were re-incubated at 35 °C for 14-18 h, and the inhibition halos around the spots were measured (mm) as indicators of antibacterial activity (Lewus & Montville, 1991). The antagonistic activity of *W. cibaria* CV4 was classified based on the halo/colony ratio: < 1 as nonantagonistic (-), > 1 as regular activity (+), > 2 as good activity (++), and > 3 as excellent activity (+++).

2.3 Evaluation of the chemical nature of the inhibitory substances

The methodology proposed by Lewus & Montville (1991) and adapted by Santos et al., (2015) was used to assess the sensitivity of inhibitory substances to proteases. The same procedure described in the previous section was repeated with some modifications. Before pouring the semi-solid BHI onto the Petri dish, 5 μL of aqueous solutions of proteinase K (20 mg/mL), amylase (20 mg/mL), and pepsin (20 mg/mL) were added next to the LAB spot/colony. Plates were incubated at 35 °C for an additional two hours. Subsequently, 7 mL of semi-solid BHI broth containing the indicator pathogen was poured over the plates. The absence of an inhibition halo in the area where the protease was applied (half-moon shape) was considered indicative of a proteinaceous origin of the inhibitory substance. A control plate without enzymes was used as a negative control, and the test was performed in triplicate.

2.4 Growth at different temperatures

To assess growth capacity at different temperatures, freshly isolated colonies from *W. cibaria* CV4 were inoculated into 1 mL of MRS broth and incubated at 35 °C for 12 h. Subsequently, 5 μL of the bacterial suspension were added at equidistant points on MRS agar plates, in duplicate. The plates were then incubated at 35, 43, and 48 °C, respectively. After 48 h, bacterial growth at the inoculation points was recorded as present (+) or absent (-).

2.5 Evaluation of antimicrobial susceptibility

Antimicrobial susceptibility tests were conducted using the disk diffusion method proposed by Bauer et al. (1966), with modifications made by dos Santos et al. (2015). *W. cibaria* CV4 was reactivated on MRS agar at 37 °C for 24 h to obtain isolated colonies. After growth, three colonies were collected, inoculated into MRS broth, and incubated for 24 h at 37 °C. Subsequently, 100 μL of the culture was inoculated onto Petri dishes containing 25 mL of MRS agar and spread evenly using a Drigalski loop. Nine disks containing the following antimicrobials (Oxoid®, Basingstoke, England) were then placed on the agar: penicillin G (10 μg), ampicillin (10 μg), vancomycin (30 μg), gentamicin (10 μg), streptomycin (10 μg), tetracycline (30 μg), chloramphenicol (30 μg), erythromycin (15 μg), and cotrimoxazole (1.25 μg of trimethoprim and 23.75 μg of sulfamethoxazole). The plates were incubated aerobically for 24 h at 35 °C. To evaluate the results, the inhibition zones (mm) were measured using a millimeter ruler, and *W. cibaria* CV4 was classified as sensitive or resistant for each antimicrobial, as proposed by Charteris et al. (1998).

2.6 Detection of antibiotic resistance genes

W. cibaria CV4 was evaluated for the presence of the following antibiotic resistance genes in its genome: *erm*B, *tet*O, *tet*M, *tet*K, *tet*L, *tet*W, *tet*S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A, and *van*B. The detection of antibiotic resistance genes was performed via PCR following previously described protocols in the literature (Table 1).

Table 1. Primers and PCR conditions used to detect antibiotic resistance genes.

Target gene	Primer	Amplicon size (pb)	Reference
*erm*B	*erm*B 1 - CGTTTACGAAATTGG*AAC*AGGTAAAGGGC *erm*B 2 - GAATCGAGACTTGAGTGTGC	359	Lina et al. (1999)
*tet*O	*tet*O 1 - *AAC*TTAGGCATTCTGGCTCAC *tet*O 2 - TCCCACTGTTCCATATCGTCA	515	Ng et al. (2001)
*tet*M	*tet*M 1 - AGTTTTAGCTCATGTTGATG *tet*M 2 - TCCGACTATTTAGACGACGG	1862	Trzcinski et al. (2000)
*tet*K	*tet*K 1 - TATTTTGGCTTTGTATTCTTTCAT *tet*K 2 - GCTATACCTGTTCCCTCTGATAA	1159	Trzcinski et al. (2000)
*tet*L	*tet*L 1 - ATAAATTGTTTCGGGTCGGTAAT *tet*L 2 - *AAC*CAGCC*AAC*TAATGACAATGAT	1077	Trzcinski et al. (2000)
*tet*W	*tet*W 1 - GAGAGCCTGCTATATGCCAGC *tet*W 2 - GGGCGTATCCACAATGTT*AAC*	168	Aminov et al. (2001)
*tet*S	*tet*S 1 - TGG*AAC*GCCAGAGAGGTATT *tet*S 2 - ACATAGACAAGCCGTTGACC	740	Obioha et al. (2023)
*aac*A-*aph*D	*aac*A-*aph*D 1 - TAATCCAAGAGCAATAAGGGC *aac*A-*aph*D 2 - GCCACACTATCATA*AC*CACTA	228	Strommenger et al. (2003)
*mec*A	*mec*A 1 - TGGCTATCGTGTCACAATCG *mec*A 2 - CTGG*AAC*TTGTTGAGCAGAG	310	Vannuffel et al. (1995)
*bla*Z	*bla*Z 1 - AAGAGATTTGCCTATGCTTC *bla*Z 2 - GCTTGACCACTTTTATCAGC	517	Vesterholm-Nielsen et al. (1999)
*van*A	*van*A 1 - TCTGCAATAGAGATAGCCGC *van*A 2 - GGAGTAGCTATCCCAGCATT	377	Lemcke & Bülte (2000)
*van*B	*van*B 1 - GCTCCGCAGCCTGCATGGACA *van*B 2 - ACGATGCCGCCATCCTCCTGC	529	Lemcke & Bülte (2000)

2.7 Cell Membrane Characteristics

2.7.1 Hydrophobicity

The hydrophobicity of *W. cibaria* CV4 was assessed using the microbial adhesion to hydrocarbons (MATH) method as described by Otero et al. (2004). Isolated colonies were subcultured three times in MRS broth at 35 °C for 12 h, and the third culture was centrifuged at 2000 *g* ×10 min. The supernatants were discarded, and the pellets were washed three times with sterile saline and resuspended to achieve an initial optical density (ODi) between 0.4 and 0.6 (at 600 nm). Subsequently, aliquots of 3.6 mL of the bacterial suspensions were transferred to sterile Falcon tubes, and 0.6 mL of hexadecane (Sigma, USA) was added to each tube. After shaking, the tubes were allowed to stand for one hour at 37 °C to facilitate phase separation. The aqueous layer was removed and transferred to new tubes for final absorbance (DOf) determination. The degree of hydrophobicity (%) was calculated according to the following equation:

$$\% \text{ hydrophobicity} = [(ODi - ODf) / ODi] \times 100$$

ODi = initial optical density
ODf = final optical density

2.7.2 Self-Aggregation

The self-aggregation capacity of *W. cibaria* CV4 was evaluated following the methodology of Ocaña & Nader-Macías (2002), with modifications. Isolated colonies were subcultured three times in MRS broth at 35 °C for 12 hours. The third culture was centrifuged at 2000 *g* × 15 min, and the supernatants were discarded. The pellets were washed three times with phosphate-buffered saline (PBS, pH 6.5) and resuspended to achieve an initial optical density (ODi) between 0.4 and 0.6 (at 600 nm). The solutions were monitored spectrophotometrically over 4 hours, with readings taken every 15 min, allowing for sedimentation during measurements to obtain the final optical density (ODf). The percentage of self-aggregation was calculated according to the following equation:

$$\% \text{ self-aggregation} = [(1 - ODf) / ODi] \times 100$$

ODi = initial optical density
ODf = final optical density

3. RESULTS

3.1 Antagonistic activity of *W. cibaria* CV4 against several bovine mastitis pathogens

The antagonistic activity of *W. cibaria* CV4 against bovine mastitis-causing pathogens is summarized in Table 2. The halo/colony ratio (Fig. 1) was calculated to determine the antimicrobial activity, and ranged from 2.86 to 4.62, indicating the lowest and highest antagonistic effects were observed against *E. faecalis* ATCC 29212 and *E. coli* O157:H7 IAL1848, respectively.

Excellent antagonistic activity (halo/colony ratio > 3) was also observed against *S. aureus* ATCC 25923, *S. agalactiae* ATCC 12386, *S. epidermidis* ATCC 12228, as well as *E. coli* O157:H7 IAL1848. Good antagonistic effects (2 < halo/colony ratio < 3) were observed against *S. uberis* ATCC 700407 and *E. faecalis* ATCC 29212.

Regarding the chemical nature of the inhibitory substances, no retraction or absence of inhibition halos was observed at the enzyme application sites of proteinase K, pepsin, or amylase for *W. cibaria* CV4.

3.2 Growth at different temperatures

After 48 hours of incubation, *W. cibaria* CV4 demonstrated growth at 35 °C (the isolation temperature), as well as at 43 °C and 48 °C, indicating resistance to elevated temperatures.

3.3 Antimicrobial susceptibility of *W. cibaria* CV4 and detection of resistance genes

Data on antimicrobial susceptibility, resistance profile, and the detection of antibiotic resistance genes are also provided in Table 2. *W. cibaria* CV4 was susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline, while it exhibited resistance to penicillin, cotrimoxazole, streptomycin, gentamicin, and vancomycin. Based on these findings, the strain was classified under susceptibility profile VI (susceptible to 4 out of 9 tested antibiotics).

The presence of the resistance genes *erm*B, *tet*O, *tet*M, *tet*K, *tet*L, *tet*W, *tet*S, *bla*Z, *mec*A, *aac*A-*aph*D, *van*A, and *van*B was investigated using PCR. No antibiotic resistance genes were detected in the screening.

Table 2. Antagonism against bovine mastitis-causing pathogens, antimicrobial susceptibility and probiotic properties of the strain *Weissella cibaria* CV4.

Properties	***Weissella cibaria* CV4**
Morphology	Rod-shaped cell
Staphylococcus aureus ATCC 25923	4.51 (+++)[1]
Streptococcus agalactiae ATCC 12386	3.28 (+++)
Staphylococcus epidermidis ATCC 12228	3.18 (+++)
Streptococcus uberis ATCC 700407	2.96 (++)
Enterococcus faecalis ATCC 29212	2.86 (++)
Escherichia coli O157:H7 IAL1848	4.62(+++)
Growth at 43° C	+
Growth at 48° C	+
Ampicillin	Susceptible
Penicillin	Resistant
Chloramphenicol	Susceptible
Cotrimoxazole	Resistant
Erythromycin	Susceptible
Streptomycin	Resistant
Gentamicin	Resistant
Tetracycline	Susceptible
Vancomycin	Resistant
Antibiotic susceptibility profile	VI[2]
Resistance genes	Not detected[3]
Hydrophobicity (%)	20.56
Self-aggregation (%)	70.50

[1]Halo/Colony Ratio: Halo/colony ratio < 1 = nonantagonistic (-); Halo/colony ratio > 1 = regular antagonistic activity (+); Halo/colony ratio > 2 = good antagonistic activity (++); Halo/colony ratio > 3 = excellent antagonistic activity (+++).

[2]Susceptibility Profile: I = susceptible to all tested antibiotics; II = susceptible to 8/9 tested antibiotics; III = susceptible to 7/9 tested antibiotics; IV = susceptible to 6/9 tested antibiotics; V = susceptible to 5/9 tested antibiotics; VI = susceptible to 4/9 tested antibiotics; VII = susceptible to 3/9 tested antibiotics; VIII = susceptible to 2/9 tested antibiotics; IX = susceptible to 1/9 tested antibiotics; X = susceptible to 0/9 tested antibiotics.

Resistance genes tested[3]: *aac*A-*aph*D, *erm*B, *tet*O, *tet*M, *tet*K, *tet*S, *tet*L, *tet*W *mec*A, *bla*Z, *van*A and *van*B.

Fig 1 Antagonistic activity of lactic acid bacteria (LAB) strains against the pathogen *Staphylococcus aureus* ATCC 25923 via the spot-on- lawn technique. The halo/colony ratio (yellow dashed line for halo; solid red line for colony) represents the antagonistic activity of the *Weissella cibaria* CV4 strain against *S. aureus*.

3.4 Cell surface characteristics

W. cibaria CV4 exhibited moderate hydrophobicity (20.56%) and strong self-aggregation capacity (70.5%).

4. DISCUSSION

In the present study, *W. cibaria* CV4 demonstrated substantial potential as an antagonistic agent against key pathogens associated with bovine mastitis. The strain exhibited notable antagonistic activity against various pathogens, with a high halo/colony ratio indicating strong inhibitory effects against *E. coli* O157:H7, *S. aureus*, *S. agalactiae*, and *S. epidermidis*. This potent antagonism against both Gram-positive and Gram-negative pathogens underscores its broad-spectrum action, making it particularly promising for applications targeting multiple mastitis-causing bacteria. The ability of *W. cibaria* CV4 to achieve a halo/colony ratio exceeding 3 for these pathogens highlights its potential efficacy as a biocontrol agent in bovine health contexts.

The antagonistic activity of LAB is likely attributed to the production of inhibitory substances such as bacteriocins, organic acids, and hydrogen peroxide, which contribute to its antimicrobial effects against specific pathogens (Steinberg et al., 2022; Yadav et al., 2022). Although bacteriocin production was not detected in the spot-on-lawn assay under the conditions tested and with the proteases applied, future studies should investigate the nature of the antimicrobial activity of *W. cibaria* CV4. These studies will include genomic analysis to elucidate the genetic basis underlying these antimicrobial mechanisms.

The ability of *W. cibaria* CV4 to grow across a wide temperature range (35 °C to 48 °C) is a valuable attribute for applications in the bovine mammary gland, where environmental conditions can vary. This thermal tolerance distinguishes *W. cibaria* CV4 from other LAB strains and suggests its robustness in challenging environments, supporting its potential use as a probiotic. This adaptability further reinforces its suitability as a biocontrol agent in dairy applications, where it can persist and maintain efficacy across diverse temperatures.

Antimicrobial susceptibility is an important criterion for selecting probiotic strains because of the risk of horizontal gene transfer of resistance markers to commensal and pathogenic members of the host indigenous microbiota (Steinberg et al., 2022). While resistance can be inherent to specific bacterial genera or species, it may also arise through mutations and the transfer of genetic material (Salam et al., 2023). Given that potential probiotic LAB could act as reservoirs for antibiotic resistance genes (Zarzecka et al., 2022), evaluating their resistance profiles is crucial to prevent the unintended spread of resistance.

W. cibaria CV4 was phenotypically susceptible to ampicillin, chloramphenicol, erythromycin, and tetracycline, and resistant to penicillin, cotrimoxazole, streptomycin, gentamicin, and vancomycin. Although resistant to 5 of the 9 antimicrobial agents tested, no resistance genes were detected by PCR under the tested conditions. However, to ensure the future safety of this strain as a probiotic, it will be essential to investigate the genetic basis of its resistance through genomic analysis, determining whether the responsible mechanisms are located within genetically mobile elements. While the risk of horizontal gene transfer of resistance cannot be ignored, probiotics with intrinsic resistance to specific antibiotics may prove beneficial for animals undergoing antimicrobial therapy by ensuring their functionality is not compromised by the administered drugs (Li et al., 2020).

The cell surface characteristics of *W. cibaria* CV4 underscore its potential as a probiotic and biofilm-forming agent. The moderate hydrophobicity suggests sufficient interaction with the mucosa, while the high self-aggregation capacity (70.5%) may facilitate biofilm formation (Espeche et al., 2009), preventing pathogen adhesion in the mammary gland. These characteristics reinforce the strain's ability to establish a stable presence, enhancing resistance to colonization by pathogenic bacteria and contributing to the formation of a protective microbial barrier. This trait is particularly valuable in preventing the colonization of mastitis-associated pathogens. Furthermore, this property holds potential for future applications, including the development of topical products or biosealers that could be directly applied to the teats of cows (Serna-Cock et al., 2019), offering an additional strategy for mastitis prevention and control.

Despite the promising potential of *Weissella* spp. as probiotics, certain strains have been identified as opportunistic pathogens capable of causing weissellosis in humans, animals, and fish (Castrejón-Nájera et al., 2018). Furthermore, *Weissella* spp. have been isolated from the milk of cows with clinical mastitis and are considered minor potential pathogen associated with this disease (Kim et al., 2023). These findings underscore the need for comprehensive investigations into the pathogenicity and virulence factors of *W. cibaria* strain CV4 to ensure its safety for potential probiotic applications.

Overall, these findings suggest that *W. cibaria* CV4 presents a multifaceted profile, characterized by high antimicrobial activity, thermal tolerance, and favorable adherence traits, positioning it as a potential candidate for further development as a biocontrol agent against bovine mastitis pathogens. Future studies should focus on investigating the genetic mechanisms of antimicrobial activity, antibiotic resistance and virulence in *W. cibaria* CV4 to assess its potential as a safe alternative approach for mastitis control.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions - CRediT

Joice Fátima Moreira Silva: Writing – original draft, Methodology, Formal analysis, Data curation, Conceptualization. **Bruna Vieira Alonso:** Investigation, Formal analysis, Conceptualization. **Paula Aparecida Azevedo Almeida:** Investigation, Formal analysis, Conceptualization. **Isabela Vieira Barbosa:** Investigation, Formal analysis. **Yasmin Neves Vieira Sabino:** Data curation, Review & editing. **Geraldo Márcio da Costa:** Conceptualization, Review & editing. **João Batista Ribeiro:** Supervision, Project administration, Funding acquisition, Conceptualization.

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FINAL CONSIDERATIONS

The findings of this thesis present a comprehensive investigation into the potential of LAB as probiotic and biocontrol agents to combat pathogens relevant to food safety and bovine mastitis. Chapter 1 addressed the essential criteria for classifying microorganisms as probiotics, highlighting requirements related to safety, functionality, and efficacy.

In Chapter 2, a collection of 84 LAB strains from various MAC producer-microregions demonstrated diverse antibiotic susceptibility profiles and antimicrobial activities. A subset of these LAB strains, particularly three lactobacilli (strains 52, 177, and 272G), showed significant antagonistic effects against *E. coli* O157:H7 and *L. monocytogenes* in milk. The implications of this study include to enhancing food safety and quality, and promoting the potential of LAB as effective natural preservatives for healthier products. Further exploration of LAB applications across different food matrices will broaden their utility in biopreservation and contribute to health promotion within the food industry.

Chapter 3 focused on 147 strains of *L. lactis* and *L. garvieae* isolated from raw milk samples, and showed good antagonistic activity against common mastitis pathogens. Among these, *L. lactis* CV151 displayed remarkable antagonistic properties, supporting its candidacy as a probiotic for bovine mastitis control. The susceptibility of this strain to antibiotics and the absence of resistance genes underscores its safety. This research highlights *L. lactis* CV151 as a possible alternative to traditional antimicrobials, warranting further investigation into its probiotic attributes to facilitate the development of a targeted therapeutic product for mastitis management.

In Chapter 4, a selection of 42 enterococci strains demonstrated significant antimicrobial potential against bovine mastitis-causing pathogens. *E. faecium* CV167 has emerged as a promising candidate, given its antagonistic activity against pathogens and the detection of bacteriocin-related genomic regions. Despite its genetic profile showing resistance and virulence genes, its potential as an antimicrobial agent remains noteworthy, underscoring the need for a rigorous safety assessment before advancing its probiotic applications. The results provide a foundation for the development of targeted probiotic treatments as sustainable alternatives to conventional antimicrobials in veterinary contexts.

Finally, Chapter 5 identified *W. cibaria* CV4 as a strain with high antimicrobial activity, thermal tolerance, and favorable adherence properties. The multifaceted profile of *W. cibaria* CV4 makes it a promising candidate for further development as a biocontrol agent against mastitis pathogens. Future studies focusing on its genetic mechanisms of antimicrobial activity, resistance, and virulence will be essential for assessing its suitability and safety in practical applications.

Overall, this thesis reinforces the value of indigenous LAB strains in both food safety and animal health contexts. These studies provide a promising foundation for developing probiotic products that could contribute to a more sustainable and effective approach to pathogen control, with potential benefits for the dairy industry and beyond. Future work should continue to explore the applications, safety, and efficacy of these LAB strains across diverse conditions and matrices, ultimately advancing the use of LAB in the development of biocontrol and probiotic interventions.