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Genotypic characterization and antimicrobial potential
of bacteria of technological interest isolated from
local dairy products of western Algeria

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Dedication

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الملخص

تهدف هذه الدراسة إلى عزل بكتيريا حمض اللاكتيك من مجموعة منتجات الألبان التقليدية و التعرف عليها باستخدام MALDITOF-MS، الكشف عن السلالات المنتجة للبكتريوسينات، وتقييم خصائصها البيوكيميائية والبيوتكنولوجية، بالإضافة إلى دراسة السلامة الحيوية لهذه السلالات.

تم عزل تسع وعشرون (29) سلالة بكتيرية من منتجات ألبان تقليدية مختلفة. أظهر التحليل بتقنية MALDITOF-MS وجود ثلاث أنواع من البكتيريا و المتمثلة في:

Enterococcus faecalis (E. faecalis) عددها 2

Levilactobacillus brevis (Lev. brevis) عددها 8

Lactocaseibacillus paracasei (L. paracasei) عددها 19

أظهر التحليل الجزئي لإثني عشر جينا مشفرا للإنتروسينات وجود الجينات التالية: في *entAS-48* و *ent1071 A/B* ثلاث عشرة عزلة ، مع وجود عزلة واحدة تحمل الجينين *entQ* و *entAS-48* معا. كما أبدت معظم العزلات نشاطا مضادا قويا للبكتيريا ضد ست سلالات بكتيرية مرجعية، حيث تراوحت أقطار التثبيط بين 10.67 ± 0.58 مم و 27.00 ± 0.00 مم، إضافة إلى نشاط متوسط ضد بكتيريا المعويات متعددة المقاومة للمضادات الحيوية (*MDR Enterobacteriaceae*)، بالإضافة إلى ذلك أظهرت جميع سلالات *Lev. brevis* و *L. paracasei* الحاملة لجينات الأنتروسين نشاطا مضادا قويا ضد البكتيريا الضارة، كما أظهر النوعان قدرة عالية على التخميض ونشاطا بروتوليتيكا قويا. بينت نتائج تقييم السلامة الحيوية أن جميع السلالات لا تحتوي على الجينات المسؤولة عن إنتاج الأمينات الحيوية وكذلك عناصر الأنتغرون، كما كشفت نتائج جينات الضراوة ومقاومة المضادات الحيوية عن وجود الجين *hyl* في السلالة *L. paracasei* S8 والحاملة كذلك للجين *entAS-48* في حين الجين *esp* لم يكن متواجد في جميع العزلات. من جهة أخرى أظهرت النتائج وجود الجين *msrA* في السلالة *L. paracasei* S93 ، والتي تحمل كذلك الجين *ent1071A/B*. أغلب العزلات لم تكن تحمل جينات الضراوة وكذلك جينات مقاومة المضادات الحيوية، مما يؤكد سلامتها.

هذه الدراسة تمثل أول تقرير علمي عن وجود جينات مشفرة للإنتروسينات في سلالات *L. paracasei* و *Lev. brevis* ، حيث تبرز النتائج الخصائص المتعددة و ملفات السلامة الملائمة لبكتيريا حمض اللاكتيك، مما يدعم إمكانية إستخدامها كمواد حافظة حيوية طبيعية و خمائر لبنية في الصناعات الغذائية، مساهمة بذلك في تحسين جودة الغذاء وحماية المستهلك.

الكلمات المفتاحية: منتجات الألبان التقليدية، *Lactocaseibacillus paracasei*، *Levilactobacillus brevis* ،
الخمائر الببوتكنولوجية، جينات الأنتروسينات، السلامة.

Abstract

The present study aimed to isolate lactic acid bacteria (LAB) from Algerian artisanal dairy products, identify them using MALDI-TOF MS, screen for bacteriocin-producing strains, evaluate their biochemical and biotechnological properties, and assess their biosafety profile. A total of twenty-nine bacterial strains were isolated from various artisanal dairy products. MALDI-TOF analysis identified three species: *Lacticaseibacillus paracasei* (*L. paracasei*, n=19), *Levilactobacillus brevis* (*Lev. brevis*, n=8), and *Enterococcus faecalis* (*E. faecalis*, n=2). Molecular screening of twelve enterocin-encoding genes revealed the presence of *entAS-48*, *entQ*, and *ent1071A/B* in thirteen isolates, with one strain harboring both *entAS-48* and *entQ* genes. All isolates tolerated acidic environment. Most isolates exhibited strong antibacterial activity against six reference bacterial strains, with inhibition zones ranging from 10.67 ± 0.58 mm to 27.00 ± 0.00 mm, and moderate activity against multidrug-resistant (MDR) *Enterobacteriaceae*. Furthermore, all *L. paracasei* and *Lev. brevis* strains carrying enterocin-encoding genes exhibited potent antimicrobial activity against the evaluated pathogenic microorganisms. Biosafety assessment showed that all strains lacked genes associated with biogenic amine production and integron elements. Screening for virulence and antibiotic resistance genes revealed the presence of the *hyl* gene in *L. paracasei* S8, while the *esp* gene was absent. The *hyl*-positive isolate also carried the *entAS48* gene. Additionally, the *msrA* gene was detected in *L. paracasei* S93, which also harbored the *ent1071A/B* gene. Overall, most isolates lacked clinically relevant virulence factors and antibiotic resistance genes, confirming their safety. This study represents the first report of enterocin-encoding genes in *L. paracasei* and *Lev. brevis* strains. The findings highlight the multifunctional properties and favorable safety profiles of these LAB, supporting their potential application as natural biopreservatives and starter cultures in food production, thereby contributing to improved food quality and consumer protection.

Keywords: artisanal dairy product, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis*, biotechnological properties, enterocin genes, safety.

Résumé

La présente étude avait pour objectif d'isoler des bactéries lactiques (BL) à partir de produits laitiers artisanaux algériens, de les identifier par MALDI-TOF MS, de cribler les souches productrices de bactériocines, d'évaluer leurs propriétés biochimiques et biotechnologiques, et d'analyser leur profil de biosécurité. Au total, vingt-neuf souches bactériennes ont été isolées à partir de différents produits laitiers artisanaux. L'analyse par MALDI-TOF MS a permis d'identifier trois espèces : *Lacticaseibacillus paracasei* (*L. paracasei*, n = 19), *Levilactobacillus brevis* (*Lev. brevis*, n = 8) et *Enterococcus faecalis* (*E. faecalis*, n = 2). La caractérisation moléculaire de douze gènes codant des entérocinés a révélé la présence des gènes *entAS-48*, *entQ* et *ent1071A/B* chez treize isolats, dont une souche portant simultanément les gènes *entAS-48* et *entQ*. Tous les isolats ont montré une tolérance aux conditions acides. La majorité des souches a présenté une forte activité antibactérienne contre six souches bactériennes de référence, avec des zones d'inhibition variant de $10,67 \pm 0,58$ mm à $27,00 \pm 0,00$ mm, ainsi qu'une activité modérée contre des entérobactéries multirésistantes (MDR). En outre, toutes les souches de *L. paracasei* et de *Lev. brevis* porteuses de gènes d'entérocinés ont montré une activité antimicrobienne marquée contre les microorganismes pathogènes testés. Les deux espèces ont présenté de fortes capacités acidifiantes et protéolytiques. L'évaluation de la biosécurité a montré l'absence de gènes impliqués dans la production d'amines biogènes ainsi que d'éléments intégrons chez toutes les souches. Le criblage des gènes de virulence et de résistance aux antibiotiques a révélé la présence du gène *hyl* chez *L. paracasei* S8, tandis que le gène *esp* était absent. La souche porteuse du gène *hyl* possédait également le gène *entAS48*. Par ailleurs, le gène *msrA* a été détecté chez *L. paracasei* S93, qui portait également le gène *ent1071A/B*. Globalement, la majorité des isolats était dépourvue de facteurs de virulence cliniquement pertinents et de gènes de résistance aux antibiotiques, confirmant ainsi leur innocuité. Cette étude constitue le premier rapport décrivant la présence de gènes codant des entérocinés chez des souches de *L. paracasei* et de *Lev. brevis*. Les résultats mettent en évidence les propriétés multifonctionnelles et le profil favorable de ces bactéries lactiques, soutenant leur potentiel d'application en tant que biopréservateurs naturels et cultures starters dans l'industrie agroalimentaire, contribuant ainsi à l'amélioration de la qualité des aliments et à la protection des consommateurs.

Mots-clés : produits laitiers artisanaux, *Lacticaseibacillus paracasei*, *Levilactobacillus brevis*, propriétés biotechnologiques, gènes d'entérocinés, biosécurité.

List of abbreviations

Aac:	Aminoglycosides
AFLP:	Amplified fragment length polymorphism
AP-PCR:	Arbitrary Primed PCR
Ace:	Aggregation substance
ARGs:	Antibiotic resistance genes
ABC:	ATP-binding cassette
Bas:	Biogenic amines
Bla:	β -lactams
BMD:	Broth microdilution
CAC :	Codex Alimentarius Commission
CFS:	Cell-free supernatant
CAD:	Cadaverine
CylA:	Cytolysin
Erm:	Erythromycin
EPS:	Exopolysaccharides
Esp :	Enterococcal surface protein
EFSA:	European Food Safety Authority
Ent:	<i>Enterocin</i>
Fca:	Fluoroquinolone
FDA:	Food and Drug Administration
GeIE:	Gelatinase
GRAS:	Generally Recognized as Safe
Hdc:	Histidine decarboxylase
HIS:	Histamine
Hyl:	Hyaluronidase
Ldc:	Lysine decarboxylase
<i>L. paracasei:</i>	<i>Lactocaseibacillus paracasei</i>
<i>Lev.brevis:</i>	<i>Levilactobacillus brevis</i>
LAB	Lactic acid bacteria
MALDI-TOF MS:	Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry.

Mdr:	Multidrug- resistance
Mcr:	Colistin
MRS:	Man, Rogosa, and Sharpe
MH:	Mueller-Hinton
MLs:	Macrolides
<i>Odc:</i>	Ornithine decarboxylase
PCA:	Plate Count Agar
PCR :	Polymerase Chain Reaction
PFGE:	Pulsed-field gel electrophoresis
PHEN:	2-phenylethylamine
PUT:	Putrescine
QNs:	Quinolones
RBCs:	Red blood cells
SPD:	Spermidine
SPN:	Spermine
<i>SPP:</i>	Subsp
Sul:	Sulfonamides
TA:	Titrateable acidity
Tet:	Tetracyclines
TSE:	Tris-Saline-EDTA buffer
TRP:	Tryptamine
TYR:	Tyramine
<i>Tdc:</i>	Tyrosine decarboxylase
Van:	Vancomycin

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General introduction

General introduction

Algerian artisanal dairy products are an important part of the country's cultural heritage and deserve preservation. They belong to a category of fermented foods characterized by rich microbial diversity, nutritional benefits, and traditional fermentation practices. These products include fresh cream, Buttermilk, Zebda, Smen, Jben, Klila, Bouhezza, Lben, and Raib. Typically, they are produced using traditional processing methods, without the addition of starter cultures or other chemical additives. The production methods of these dairy products vary not only between product types but also across regions (Boudalia *et al.*, 2020; Boumediene *et al.*, 2024; Leksir *et al.*, 2019).

Lactic acid bacteria (LAB) are the primary microorganisms responsible for their fermentation (Leksir *et al.*, 2019). The genera most frequently used in food fermentation include *Lactobacillus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*, *Pediococcus*, *Weissella*, and *Enterococcus* (Wang *et al.*, 2021). Traditional fermentation promotes the growth of a diverse microbial community, which contributes to the distinct sensory and functional characteristics of each product (Tamang *et al.*, 2020). The diversity of LAB in these products depends on several factors, including geographical origin, raw milk quality, processing practices, and environmental conditions (Quintana *et al.*, 2020; Biçer *et al.*, 2025).

Nowadays, traditional dairy products have become a major focus of scientific research due to their technological properties and functional importance. Among these properties, antimicrobial activity plays a crucial role in the selection of probiotic strains (Bouchibane *et al.*, 2023; Hadeef *et al.*, 2023). This activity is mainly attributed to the production of a wide range of metabolic compounds, including organic acids, bacteriocins, exopolysaccharides (EPS), and hydrogen peroxide (Fijan, 2023; Sepehr *et al.*, 2024). Consequently, LAB strains can inhibit a broad spectrum of foodborne pathogens and spoilage microorganisms, including multidrug-resistant (MDR) members of the *Enterobacteriaceae* family. These Gram-negative bacteria have developed resistance to antibiotics due to the inappropriate use of antimicrobial agents (Jian *et al.*, 2021a). The increasing isolation of MDR pathogens poses a major global health challenge by complicating the treatment of infections. The food chain has been identified as a key route for the transmission of antibiotic-resistant bacteria between humans and animals (Stefańska *et al.*, 2021). In particular, the presence of MDR *Enterobacteriaceae* in food systems represents a significant threat to both human and animal health, highlighting

the importance of ensuring food safety to prevent foodborne diseases (Ahmed *et al.*, 2023; Sorathiya *et al.*, 2025; Hu *et al.*, 2023).

Given that MDR represents a major global threat and is responsible for numerous severe diseases, there is an urgent need for effective alternative strategies to control and prevent infectious diseases. Currently, probiotics particularly *Lactobacillus* species and their bacteriocins have attracted considerable scientific interest due to their potential as natural antimicrobial agents capable of inhibiting pathogenic microorganisms (Bashir & Ali, 2021).

In recent years, interest in bacteriocins has grown considerably, particularly due to their potential as alternatives to conventional antibiotics in both animal and human applications (Grande Burgos *et al.*, 2014). Bacteriocins are gene-encoded antimicrobial peptides produced by bacteria. These peptides are highly diverse in their antimicrobial activity, structural characteristics, regulatory mechanisms, and associated biosynthetic gene clusters. Bacteriocin genes are often located on mobile genetic elements, such as plasmids, conjugative elements, and integrative elements, which facilitate their dissemination among bacterial species or strains through horizontal gene transfer (HGT) (Arbulu & Kjos, 2024; Martínez-Bueno *et al.*, 2006). Among these bacteriocins, enterocins produced by *Enterococcus* spp. have been extensively studied for their applications in food preservation and safety, probiotic formulations, and veterinary medicine (Burke *et al.*, 2013; Kasimin *et al.*, 2022).

LAB are widely recognized as safe (GRAS) owing to their beneficial effects on host health; however, strains harboring enterocin genes with technological relevance must undergo biosafety evaluation before their use in industrial application. These assessments should include screening for antibiotic resistance determinants, integrons, virulence-associated genes, and biogenic amine-producing capabilities. Recent evidence suggests that LAB may serve as reservoirs of antibiotic resistance genes (ARGs). Due to their extensive use in the agri-food sector and their interaction with the gastrointestinal microbiota, LAB may contribute to the horizontal transfer of ARGs. Therefore, continuous safety surveillance of LAB strains proposed as feed additives is essential to mitigate potential public health risks (Rozman *et al.*, 2023; Stefańska *et al.*, 2021).

Although traditional Algerian dairy products are recognized as reservoirs of LAB with promising antimicrobial properties, genetic variability, and functional potential (Bentahar *et al.*, 2024; Bouchibane *et al.*, 2024; Belkacem *et al.*, 2025), the safety aspects and detailed

characterization of indigenous bacteriocin-producing LAB remain inadequately explored. This lack of data, particularly regarding enterocin-producing strains, limits their safe and effective utilization in food and health-related applications.

In this regard, the present study focused on the selection and characterization of LAB strains harboring enterocin genes with strong antimicrobial activity and GRAS status, highlighting their potential relevance as alternative antimicrobial strategies. The objectives of this study were to isolate LAB from traditional dairy products made from raw cow milk in western Algeria and to identify and characterize novel autochthonous bacteriocin-producing LAB strains, with particular emphasis on non-genus-specific bacteriocin-encoding genes. In addition, the biochemical characteristics of the selected strains were determined, and their technological properties including acidifying, proteolytic, and lipolytic activities were evaluated. Their antimicrobial activity against MDR foodborne pathogens and reference (ATCC) strains was also assessed. Finally, the safety of the selected LAB strains was evaluated by screening for antibiotic resistance determinants, virulence genes, biogenic amine-encoding genes, and integron-associated genes.

This thesis is presented in the form of an article-based thesis and is structured into three main parts. The first part is devoted to a literature review, organized into two major chapters: the first focusing on artisanal dairy products and lactic acid bacteria, and the second on bacteriocins and the safety assessment of these strains. The second part comprises the materials and methods, presented in the form of original research articles, either published or submitted. The main findings are then outlined in a general discussion. Finally, a general conclusion, including some perspectives, is presented.

Literature review

1. The Dairy Sector in Algeria: Economic Importance and Current Challenges

The dairy sector represents one of the most strategic components of the Algerian agri-food economy because of its essential role in ensuring food security, improving nutrition, generating rural employment, and supporting agricultural development (Meklati *et al.*, 2020 ; Mamine *et al.*, 2021;). Algeria is the largest milk consumer in North Africa, with annual per capita consumption estimated at approximately **110–120 L**, which is considerably higher than the African average (Djermoun & Chehat, 2012). National demand for milk is estimated at **5–6 billion liters of milk equivalent per year**, whereas domestic production satisfies only **55–65%** of total demand, resulting in a persistent supply deficit (Meklati *et al.*, 2020). Consequently, Algeria remains among the world's largest importers of dairy products, particularly skimmed and whole milk powder, importing approximately **300,000–450,000 tonnes annually**, depending on domestic production and international market conditions (Mamine *et al.*, 2021). The dairy import bill frequently exceeds **USD 1 billion per year**, making dairy products one of the country's largest food import categories (Mamine *et al.*, 2021). To reduce this dependence, the Algerian government has implemented several support policies, including subsidies for raw milk production, incentives for milk collection centers, veterinary and artificial insemination programs, and financial support for dairy farmers and processors (Mamine *et al.*, 2021). Despite these efforts, the sector continues to face major structural constraints, including low dairy cow productivity, limited forage availability, dependence on imported feed, fragmented farm structures, inefficient milk collection systems, and weak coordination among actors along the dairy value chain (Djermoun & Chehat, 2012 ; Meklati *et al.*, 2020). Therefore, enhancing the productivity, competitiveness, and sustainability of the Algerian dairy sector remains a key priority for achieving greater food security and reducing dependence on international dairy markets (Mamine *et al.*, 2021).

2. Artisanal dairy products

2.1. Background

In recent years, there has been growing interest in foods closely associated with specific regions or territories. Consumers are increasingly drawn to local products that carry a traditional identity. Fermented foods have been consumed since antiquity and continue to be an essential part of diets worldwide. In Algeria, artisanal dairy products constitute a significant component of the national diet and represent a valuable gastronomic heritage that

deserves preservation. These products include Buttermilk, Fresh cream, Jben, Smen, Lben, Zebda, and Raib (Leksir *et al.*, 2019a).

According to the Codex Alimentarius Commission (CAC) standard CXS 243-2003, fermented milks are defined as milk products obtained through the fermentation of milk by specific microorganisms, resulting in a reduction of pH, with or without other permitted ingredients. The action of microorganisms lowers the pH, sometimes resulting in coagulation. Additionally, starter cultures must remain viable, active, and abundant in the fermented product until the date of minimum durability. If the product is heat-treated after fermentation, the requirement for live microorganisms no longer applies. Fermented dairy products are typically produced through the activity of LAB, which are considered sources of probiotic-like microorganisms (Bintsis&Papademas, 2022a).

Traditional dairy products are made using livestock milk and ancestral production techniques, and their variety depends on the type of milk used, such as cow, goat, sheep, or camel. They can be made from cow's milk alone or mixtures of different milks. Milk from various animals differs in chemical composition, such as fat, protein, total solids, mineral content, and lactose. Manufacturing practices affect various parameters of the final products, including their physical, chemical, sensory, and nutritional properties. Moreover, the main factors influencing milk processing are geo-climatic conditions, animal health, breed, feed, season, and stage of lactation (Figure1). Fermented dairy products can be classified according to several factors, including the type of fermentation (involving lactic acid bacteria alone or in combination with fungi or other adjunct cultures), the mode of coagulation (enzyme-induced, acid-induced, or acid combined with heat), and the extent of whey separation (ranging from none to complete) (Leksir *et al.*, 2019b; Bintsis&Papademas, 2022b).

Moreover, these products are widely used in various culinary preparations, such as Couscous, El Merdoukh, R'fiss, Trida, Chakhchoukha, Makrout, and Baklawa. In Algeria, yogurt is the most widely consumed dairy product, followed by cheese and dairy desserts. In contrast, rancid butter and fresh cream are the least consumed (Bousbia *et al.*, 2017; Benkirane *et al.*, 2022a).

Milk is well known for its nutritional value and associated health benefits (Figure 3). However, these same nutrient-rich components also make milk highly perishable.

Fermentation is therefore regarded as one of the most effective traditional methods for preserving milk and extending the shelf life of its nutrients (Figure 2)(Tamang et al., 2020).

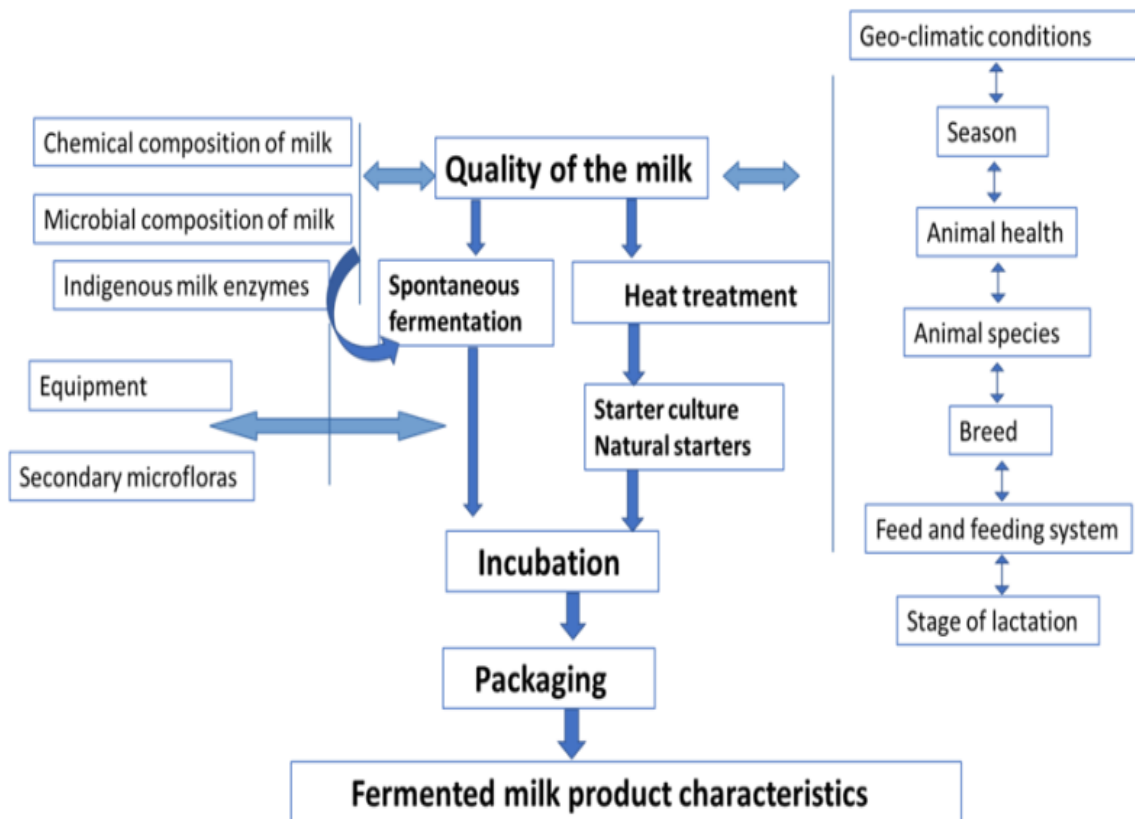


Figure 1. Main factors affecting the properties of fermented milk products (Bintsis & Papademas, 2022b).

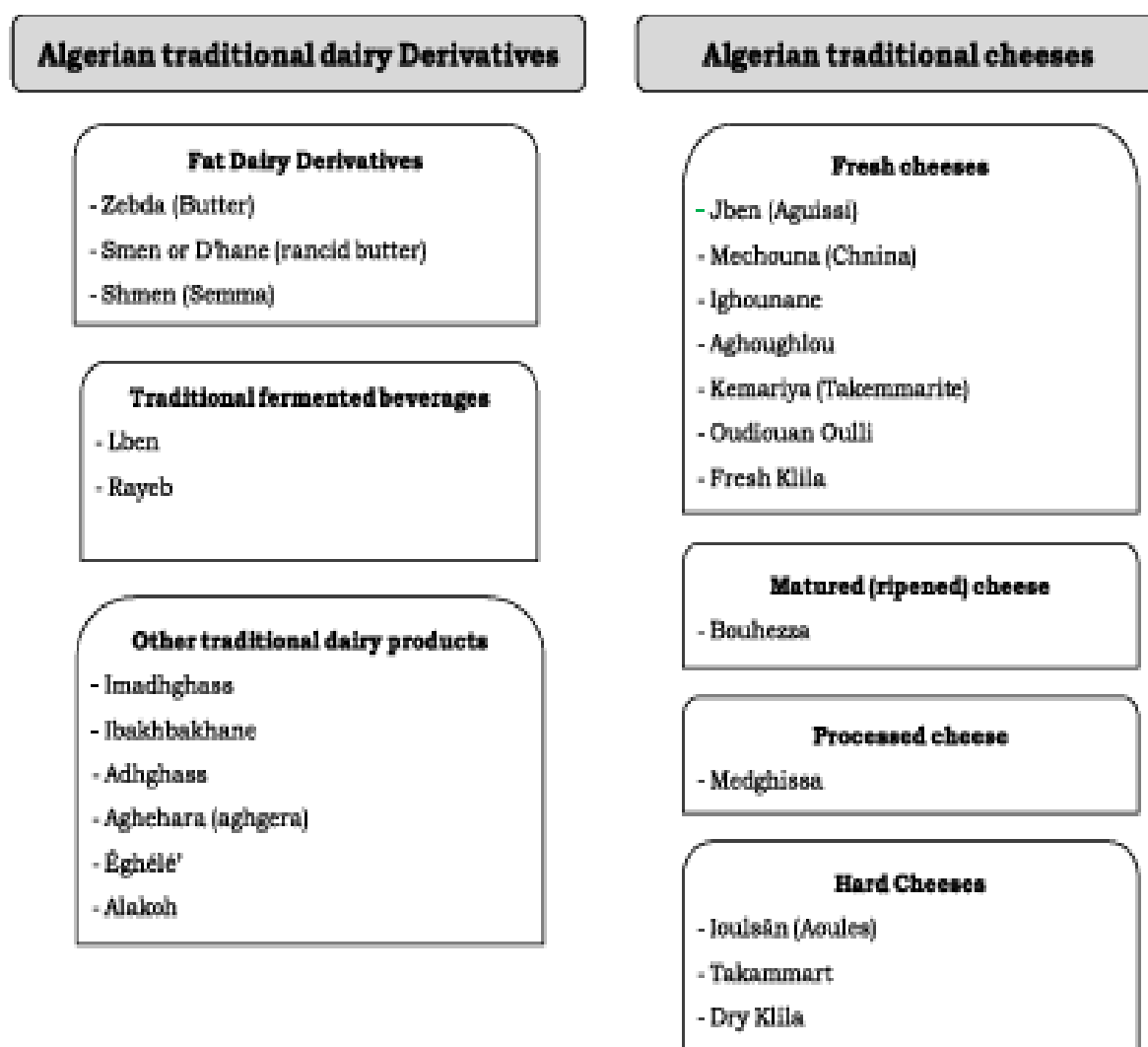


Figure 2. Algerian artisanal dairy products (Leksir et al., 2019c).

1.2. Main types of Algerian traditional dairy products

Algeria has a rich tradition of dairy products that reflect its diverse cultural influences. Among these products are cheese, butter (Zebda), Smen (Dhan), Lben, Rayeb, and fresh cream. The various Algerian fermented dairy products are shown in Figure 2.

1.2.1. Cheeses: Traditional cheeses in Algeria are typically produced on small farms using raw milk from indigenous animal breeds, often without adding commercial or natural starter cultures. Production historically relied on spontaneous fermentation driven by the natural microflora in the milk, so the quality of the final cheese depended largely on milk hygiene and its microbial population. LAB play a key role by enhancing food safety through the production of bacteriocins and lactic acid, as well as by contributing to flavor, aroma, and

accelerated cheese maturation (Benheddi&Hellal, 2019). Among the traditional cheeses produced in various regions of Algeria are Jben, Klila, Mechouna, Bouhezza, Takammérite, Madghissa, Igounanes, and Aoules (Boudalia *et al.*, 2020; Medjoudj *et al.*, 2020; Benamara *et al.*, 2016; Benyahia *et al.*, 2025).

1.2.2. Lben: traditionally, it is obtained from cow's milk, and in smaller quantities from sheep's, goat's, or camel's milk. The raw milk is placed in a Rawaba and kept at room temperature for 24 to 72 hours until coagulation occurs. After coagulation, the product is called Rayeb. The Rayeb is then shaken for 30 to 40 minutes, resulting in the separation into two products: Zebdabeldiya (butter) and Lben (fermented buttermilk) (Leksir *et al.*, 2019b; Benkirane *et al.*, 2022).

1.2.3. Rayeb: is a fermented milk obtained through the spontaneous fermentation of raw milk at room temperature, over a period of 12 to 72 hours, depending on the season. The resulting coagulum may be consumed as is or further processed (Leksir *et al.*, 2019b; Boussekine *et al.*, 2020).

1.2.4. Fresh cream: is a fermented dairy product composed primarily of concentrated milk fat, in which the fat globules are stabilized by surrounding phospholipid–protein membranes that prevent coalescence. This product is often paired with other dairy items like fresh cow's milk cheese, used in butter production, or as an ingredient in various culinary dishes. Artisanal sour cream is traditionally produced through the spontaneous fermentation of raw milk at the household level. In the absence of homogenization, the milk fat naturally rises to the surface due to density differences among milk constituents, forming sour cream (Bendelja Ljoljić *et al.*, 2025).

1.2.5. Butter (Zebda): is obtained by churning *Rayeb*. Churning may be performed using traditional tools such as the *Chekoua* (a manually operated goatskin bag) or modern equipment, including electric churns with metal, wooden, or plastic paddles, as well as simple plastic bottles. At the end of churning, a small amount of warm water (40–50 °C) is added to promote fat globule aggregation and improve butter yield. Churning time depends on ambient temperature, the equipment used, and the quantity and fat content of *Rayeb*, which varies according to the animal species producing the milk. The butter is collected by hand or with a strainer and washed several times to remove residual *Lben* (buttermilk) (Boussekine *et al.*,

2020;Leksir *et al.*, 2019c).

1.2.6. Smen: also known as *Dhan*, is an artisanal fermented and clarified butter highly valued by Algerian families. After churning, the butter may be processed immediately into Smen or stored until a sufficient quantity is accumulated. The fresh butter is either washed with warm water and salted or melted and heated, after which herbs or spices may be added. The production process of Smen varies by region (Figure 4). This fermented product is appreciated for its distinctive flavor and aroma, as well as its long shelf life (Boussekine *et al.*, 2020).

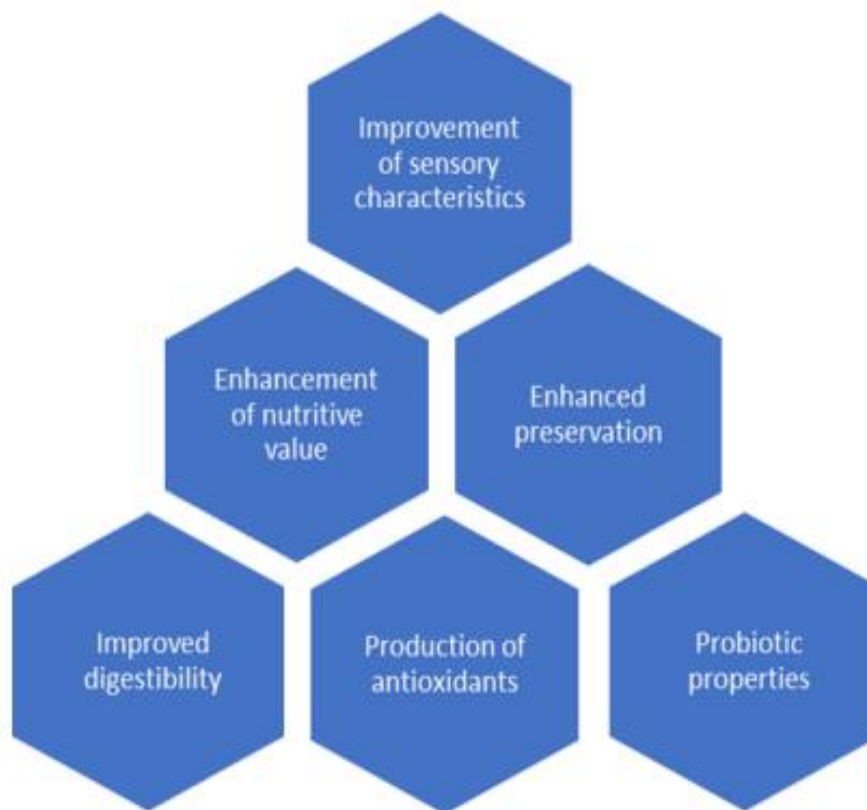


Figure 3. Benefits health of dairy products (Bintsis&Papademas, 2022c).

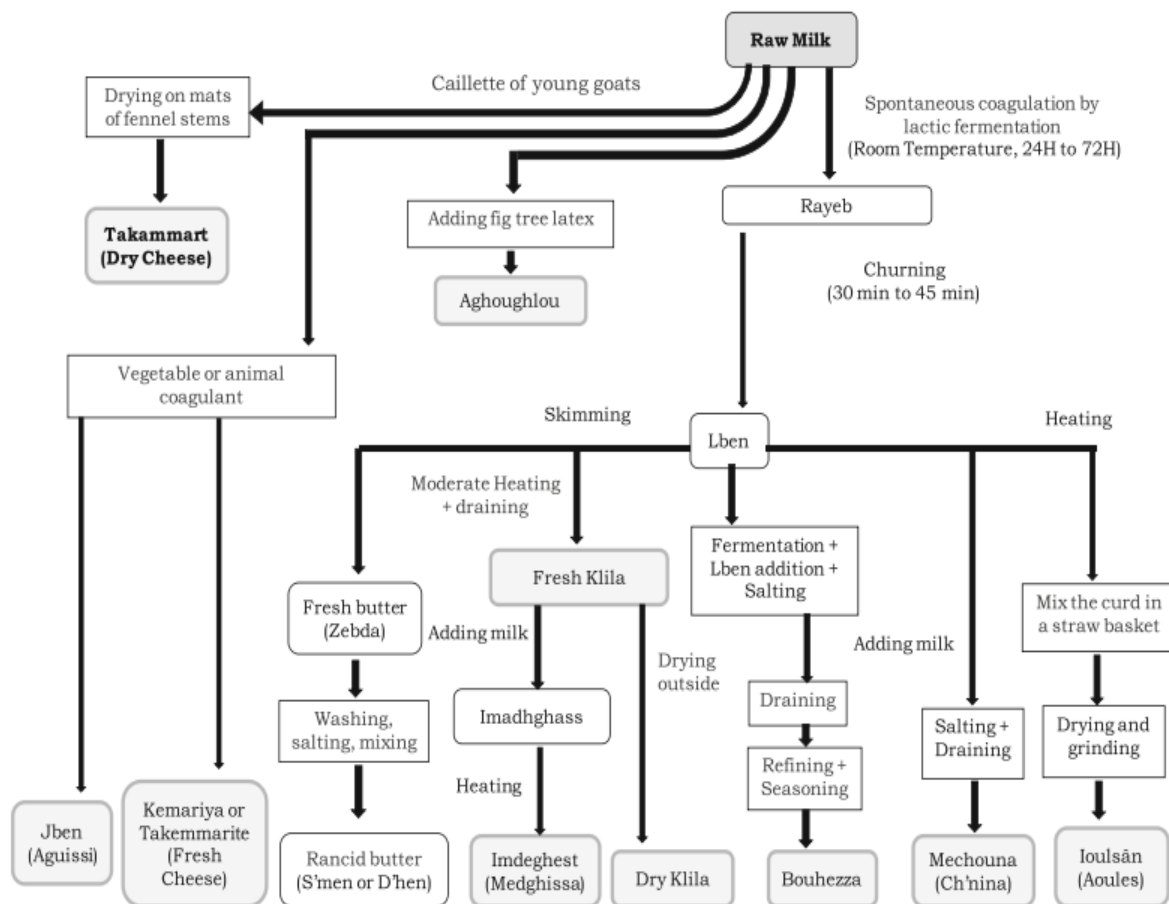


Figure 4. Different steps of Smen/Dhan process (Leksir et al., 2019c).

3. Lactic Acid Bacteria

2.1. Background

The concept of LAB was developed at the beginning of the 1900s, when they were traditionally referred to as milk-souring organisms (Liu *et al.*, 2014). LAB are historically defined as a heterogeneous group of microorganisms. They are ubiquitous and can be found in fermented foods rich in carbohydrate substrates, including fermented meat, fish, beets, plants, potatoes, vegetables, fruits, and dairy products. In addition, LAB are also found in non-fermented environments such as the intestinal tract, mouth, respiratory tract, and genital tract of humans and animals (Adams, 1999; König *et al.*, 2009).

LAB are a group of Gram-positive, catalase-negative bacteria that typically appear as rods or cocci and exhibit strong tolerance to low pH conditions, although pseudo-catalase activity has been observed in cultures grown under low-sugar conditions. To date, more than 60 genera of LAB have been identified, including *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, and *Streptococcus*, all belonging to the phylum Firmicutes. In contrast, *Bifidobacterium*, which is often functionally associated with LAB, belongs to the phylum Actinobacteria (Wang *et al.*, 2021a).

LAB are classified into two groups based on their fermentation pathways: homofermentative and heterofermentative. Homofermentative LAB produce lactic acid as the primary end product of glucose fermentation, with *Lactococcus* and *Lactobacillus* as typical examples. Heterofermentative LAB generate lactic acid along with carbon dioxide and acetic acid via the pentose phosphate (phosphoketolase) pathway, with representative genera including *Leuconostoc*, *Weissella*, and *Oenococcus*. Notably, heterofermentative LAB are widely used in the dairy industry because they contribute to flavor development through the production of aroma-enhancing compounds (Carr *et al.*, 2002).

Recently, the application of LAB in the food industry has gained great economic importance due to their many valuable characteristics. They can be used alone or in combination with other starter microorganisms, such as yeasts, bacteria, and fungi, to achieve a uniform and high-quality end product (De Vuyst&Vandamme, 1994).

2.2. Taxonomy of LAB genera

Taxonomy is the discipline concerned with the description, identification, nomenclature, and classification of organisms (Rahkila, 2015). The biodiversity of LAB depends on their taxonomic diversity at the genus, species, and strain levels, as well as their genetic and phenotypic variability. This diversity is reflected in their association with a wide range of habitats and ecosystems. Taxonomic studies have revealed a broad diversity of LAB, comprising the following families, as bellow (Holzapfel& Wood, 2014).

2.2.1. *Lactobacillus*

It is the most heterogeneous genus among LAB. This heterogeneity is reflected in the wide range of DNA base compositions (mol% G+C) observed among species within the genus. *Lactobacillus* includes numerous species exhibiting considerable diversity in

phenotypic, physiological, and biochemical characteristics. Traditionally, the genus was divided into three subgenera: *Thermobacterium*, *Streptobacterium*, and *Betabacterium* based on optimal growth temperatures and hexose fermentation pathways. Additionally, *Lactobacillus* species are classified into three metabolic groups: Group I (obligate homofermentative species), Group II (facultative heterofermentative species), and Group III (obligate heterofermentative species). Molecular identification based on 16S rRNA gene analysis further supports the following principal groupings:

- The *Lactobacillus delbrueckii* group, primarily comprising homofermentative species;
- The *Lactobacillus casei*–*Pediococcus* group, which includes obligate homofermentative species as well as facultative and obligate heterofermentative species; and
- The *Leuconostoc* group, which contains some obligate heterofermentative species.

2.2.2. *Carnobacterium*

The genus *Carnobacterium* was originally isolated together with *Lactobacillus* species and initially classified as group III lactobacilli, which included *Lb. divergens*, *Lb. carnis*, and *Lb. piscicola*. It was later separated from *Lactobacillus* based on its homofermentative metabolism, its ability to grow at high pH (e.g., pH 9) and low temperatures, and differences in fatty acid composition. The distinction between *C. piscicola* and *C. divergens* was confirmed by DNA–DNA hybridization. Currently, the genus *Carnobacterium* comprises 11 recognized species. These bacteria are widely found in foods, including dairy and meat products, and play an important role in the biopreservation of food.

2.2.3. *Enterococcus*, *Leuconostoc*, *Lactococcus*, *Streptococcus*

The genus *Enterococcus* was described by Schleifer and Kilpper-Bälz in 1984. Historically, it was not widely used in food technology, particularly *E. faecalis* (formerly *Streptococcus faecalis*), due to its association with pathogenicity. Currently, the genus comprises 46 recognized species. Molecular characterization using 16S rRNA gene sequencing has identified several phylogenetic groups, including the *E. faecium*, *E. faecalis*, *E. avium*, *E. casseliflavus*, *E. dispar*, *E. saccharolyticus*, and *E. cecorum* groups. *Enterococcus* species are widely applied in poultry nutrition, and notably, *E. faecalis* and *E. faecium* are also used as probiotics for both animals and humans.

The genus *Leuconostoc* was described by van Tieghem in 1878. Phenotypically, it

consists of heterofermentative, coccoid LAB that produce D-lactic acid from glucose and do not generate ammonia from arginine. *Leuconostoc* species are capable of growth at low temperatures and are widely used in the dairy industry due to their ability to produce diacetyl from citrate in milk. They are also commonly found in meat and other fermented foods. Phylogenetically, the genus is divided into three evolutionary branches: the *L. mesenteroides*, *L. lactis*, and *L. gelidum* species groups.

The genus *Lactococcus* is primarily associated with dairy products, although only *Lactococcus lactis* is widely used in dairy technology. Species of this genus are also found in other fermented foods, dairy environments, plants, and animal sources. *Lactococcus* belongs to the family Streptococcaceae and is closely related to species in the genus *Streptococcus*. Currently, the genus comprises 12 recognized species.

The genus *Streptococcus* was first described by Rosenbach in 1884. Based on morphological, serological, physiological, and biochemical characteristics, it includes a wide range of organisms, such as *S. agalactiae*, the intestinal Group D streptococci (*S. faecalis*, *S. faecium*), and Group N starter bacteria (*S. cremoris*). The genus currently comprises more than 50 recognized species. Notably, *S. thermophilus* is frequently used in combination with *Lactobacillus delbrueckii* subsp. *bulgaricus*, *L. lactis*, and/or *L. helveticus* as a starter culture for yogurt production. A well-established symbiotic relationship exists between *S. thermophilus* and *L. delbrueckii* subsp. *bulgaricus*: *S. thermophilus* produces formic acid, which stimulates the growth of lactobacilli, while the lactobacilli contribute flavor compounds, such as acetaldehyde, and provide proteolytic activity that supports the growth of *S. thermophilus* in milk.

2.2.4. Bifidobacterium

The genus *Bifidobacterium* belongs to the phylum Actinobacteria and currently comprises 44 recognized species and 9 subspecies. Phylogenetic analyses have identified several groups within the genus, including the *B. adolescentis*, *B. pullorum*, *B. boum*, and *B. pseudolongum* groups. *Bifidobacterium* species play an important role in food microbiology and human nutrition due to their production of inhibitory compounds, such as bacteriocins and organic acids, which contribute to food preservation.

2.2.5. *Pediococcus*, *Aerococcus*

The genus *Pediococcus* was previously defined as a heterogeneous, acidophilic, catalase-negative group of homofermentative LAB, although some strains produce pseudo-catalase. These bacteria divide in two perpendicular planes to form tetrads. *Pediococcus* species are widely used in food technology, particularly as starter cultures for cheese production. Molecular studies indicate that their DNA has a G+C content ranging from 34 to 44 mol%. Taxonomically, the genus comprises 11 recognized species, including *P. halophilus*, *P. acidilactici*, and *P. pentosaceus*.

The genus *Aerococcus* consists of Gram-positive, facultatively anaerobic, non-spore-forming, catalase-negative coccoid bacteria with a relatively low G+C content (35–43%). This genus is of minor interest in food technology and currently comprises seven recognized species. Phenotypically, *Aerococcus viridans* is very similar to *Pediococcus urinaeaequi*, and phylogenetic studies using DNA–DNA hybridization have demonstrated intragenetic relationships between these two species.

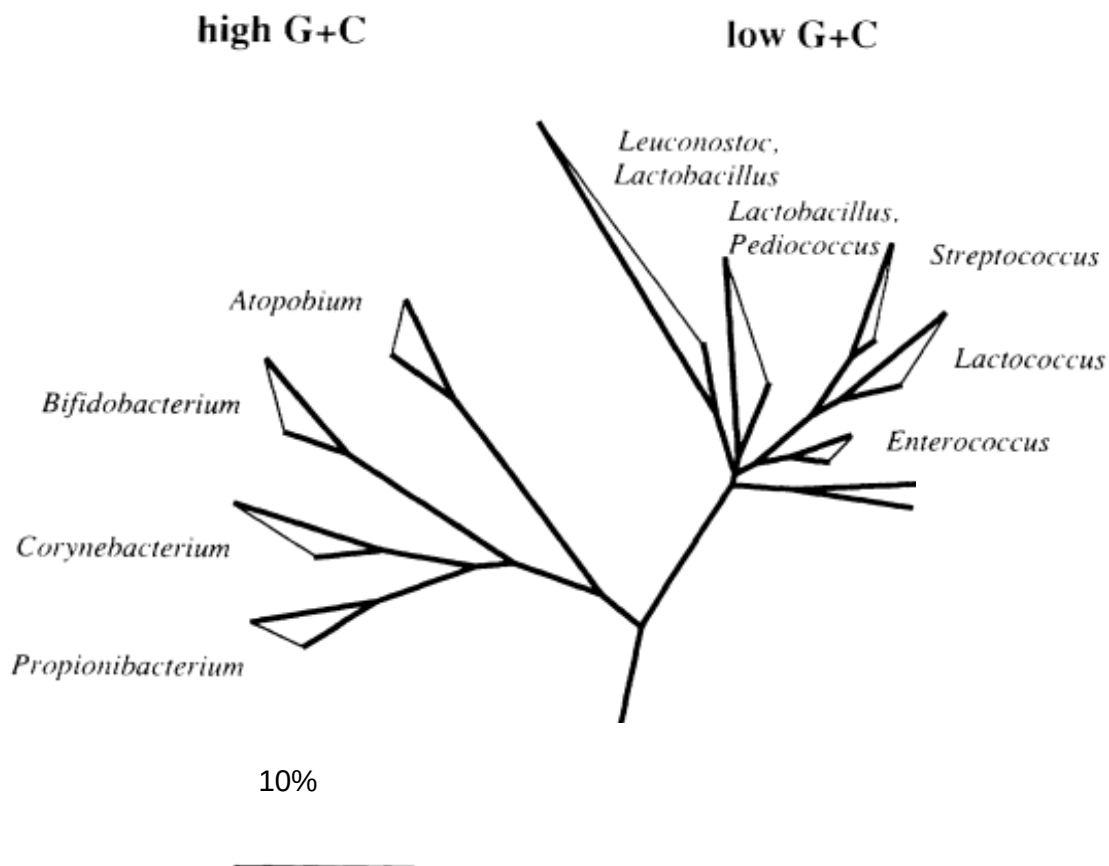


Figure 5. Phylogenetic tree of LAB based on comparative 16S rRNA sequence analysis, showing phylogenetic groups with low and high G+C content in their DNA (Schleifer and Ludwig, 1995).

2.3. Taxonomic Reclassification of LAB

Traditionally, LAB were classified according to phenotypic, physiological, and biochemical characteristics, including cell morphology, carbohydrate fermentation profiles, growth temperature, and metabolic end products (Stiles & Holzapfel, 1997). However, advances in molecular phylogeny, whole-genome sequencing, and comparative genomics have substantially reshaped the taxonomy of this bacterial group (Salvetti *et al.*, 2018). Currently, LAB are assigned to the order Lactobacillales, within the class Bacilli and the phylum Bacillota (formerly *Firmicutes*) (Parte *et al.*, 2020). This order comprises several families, including Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Enterococcaceae, Carnobacteriaceae, and Aerococcaceae (Zheng *et al.*, 2020). A major taxonomic revision proposed by Zheng *et al.* (2020), based on phylogenomic analyses of more than 260 genomes, demonstrated that the former genus *Lactobacillus* was highly polyphyletic. Consequently, it was reorganized into 25 genera, including *Lacticaseibacillus*, *Ligilactobacillus*, *Latilactobacillus*, *Levilactobacillus*, and *Liquorilactobacillus*, while retaining a redefined genus *Lactobacillus*. This genome-based reclassification provides a more accurate representation of the evolutionary relationships among LAB and has become the accepted taxonomic framework for contemporary microbiological, food, and probiotic research (Zheng *et al.*, 2020; Huang *et al.*, 2021).

2.4. Methods used for the characterization of LAB

For many years, several methods have been used for the detection and identification of LAB populations in artisanal dairy products, including both phenotypic and genotypic approaches. Phenotypic techniques are typically employed for preliminary characterization and are based on morphological, biochemical, and physiological tests. The molecular approach is a crucial method for bacterial taxonomy and classification (Sharma *et al.*, 2020a).

2.4.1. Phenotypic identification

2.4.1.1. Morphological method

This technique involves both macroscopic and microscopic examination of bacteria. Macroscopically, colonies are observed on culture media to assess their shape, color, size, texture, and other visible characteristics. Microscopically, Gram staining is performed to determine the bacterial cell's shape, arrangement, Gram reaction, and the presence or absence of structures such as endospores. Together, these observations provide essential information for the preliminary identification and characterization of bacterial species (Salminen *et al.*, 2004).

2.4.1.2. Biochemical and physiological methods

Early classification of LAB was based on physiological and biochemical characteristics. Physiological identification includes tests such as glucose fermentation products, hydrolysis of aesculin and arginine, nitrate reduction, growth at different temperatures, pH tolerance, NaCl concentration, and oxygen tolerance. Biochemical characterization focuses on determining the presence or absence of *meso*-diaminopimelic acid in the cell wall, which serves as a key taxonomic marker. Cell wall composition forms the basis for several classical taxonomic characteristics. Gram-positive bacteria show considerable variation in the composition and structural arrangement of their peptidoglycan, which is considered an important taxonomic criterion (Schleifer&Kandler, 1972).

2.4.2 Genotypic characterization

Several molecular methods are commonly employed for the identification and characterization of LAB, including MALDI-TOF MS, 16S rRNA gene sequencing, randomly amplified polymorphic DNA (RAPD), and pulsed-field gel electrophoresis (PFGE). These techniques provide high-resolution discrimination between closely related strains and complement traditional phenotypic methods for accurate species-level identification (Randazzo *et al.*, 2009).

2.4.2.1. Matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF / MS)

MALDI-TOF MS is a modern molecular proteomic technology used for the characterization and identification of bacteria at the genus and subspecies levels, based on the protein composition of microbial cells (Cherkaoui *et al.*, 2011). It is rapid, accurate, and cost-effective and can be applied directly to crude bacterial extracts, whole cells, or simple cell

lysates. The technique has also been used to characterize yeasts and fungi. It is applied in medical diagnostics, biodefense, environmental monitoring, and food quality control. Additionally, MALDI-TOF has been developed for general protein analysis after isolation and purification (Lay, 2001).

2.4.2.2. PCR and 16S rRNA Gene Sequencing

This technique amplifies a gene or gene fragment from a small number of cells, followed by DNA sequencing. Typical amplification conditions include an initial denaturation at 94°C, annealing at 45°C, and elongation at 72°C (Salminen & Wright, 1998; Nieto-Arribas *et al.*, 2009). Sequencing of the 16S ribosomal RNA (rRNA) gene is considered a universal method for bacterial taxonomy because it contains conserved regions common to all microorganisms and variable regions that differ between species (Regueira-Iglesias *et al.*, 2023). This molecular approach is rapid, automated, and reliable for characterizing various LAB species isolated from food samples (Patel, 2001a). Both 16S and 23S rRNA sequencing can be used to identify LAB, often to the species level, including *Lactobacillus paracasei*, *Lactobacillus plantarum*, and *Leuconostoc mesenteroides* (Callon *et al.*, 2004).

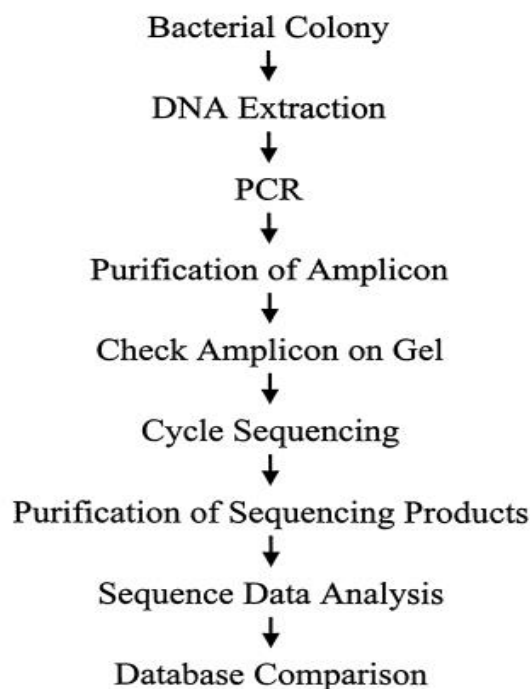


Figure 6. Steps of sequence identification (Patel, 2001).

2.4.2.3. Randomly amplified polymorphic DNA (RAPD)

RAPD is a PCR-based technique widely used to develop DNA markers and assess genetic polymorphism within and between species. It is an effective tool for evaluating genetic diversity, resolving taxonomic relationships, and identifying genetic variation among individuals and populations by detecting mutations in both coding and non-coding regions of the genome. RAPD markers are generated by amplifying anonymous genomic regions using single, short arbitrary primers (8–12 nucleotides), which eliminates the need for prior sequence information. The technique, developed independently as RAPD and AP-PCR (Arbitrary Primed PCR), is considered simple, rapid, and cost-effective, making it suitable for laboratories beginning molecular work. It has been widely applied in genetic mapping, marker development for desirable traits, population and evolutionary genetics, and plant and animal breeding (Bardakci *et al.*, 2000; Dilipan *et al.*, 2017).

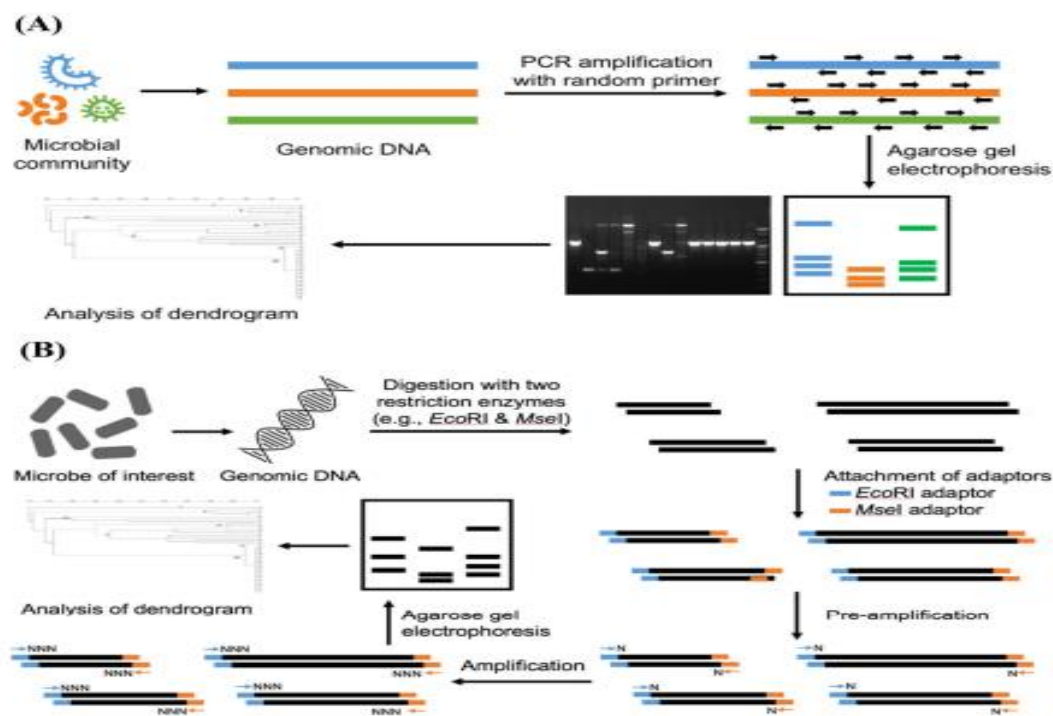


Figure 7. Schematic diagram for random amplified polymorphic DNA (A) and amplified fragment length polymorphism (B) (Sharma *et al.*, 2020b).

2.4.2.4. Pulsed-field gel electrophoresis (PFGE)

PFGE is a technique used to fractionate high-molecular-weight DNA ranging from approximately 10 kb to 10 Mb. Unlike conventional electrophoresis, PFGE applies an alternating electric field in different orientations, enabling the separation of very large DNA

molecules that would otherwise migrate as a single band. PFGE technique has been developed by Schwartz and Cantor in 1984 and it has become an essential tool for genome analysis and epidemiological typing (Lopez-Canovas *et al.*, 2019).

PFGE allows the manipulation and analysis of intact chromosomes or large chromosomal fragments, supporting the construction of physical genome maps and the localization of cloned sequences. It provides excellent resolution for differentiating bacterial isolates, including *Staphylococcus aureus*, vancomycin-resistant *Enterococcus*, *Streptococcus pyogenes*, *Pseudomonas aeruginosa*, *Listeria monocytogenes*, *Escherichia coli*, *Klebsiellapneumoniae*, *Vibrio cholerae*, and others. This technique has several advantages, including strong discriminatory power, high reproducibility, typeability, and relative ease of use (Nassonova, 2008).

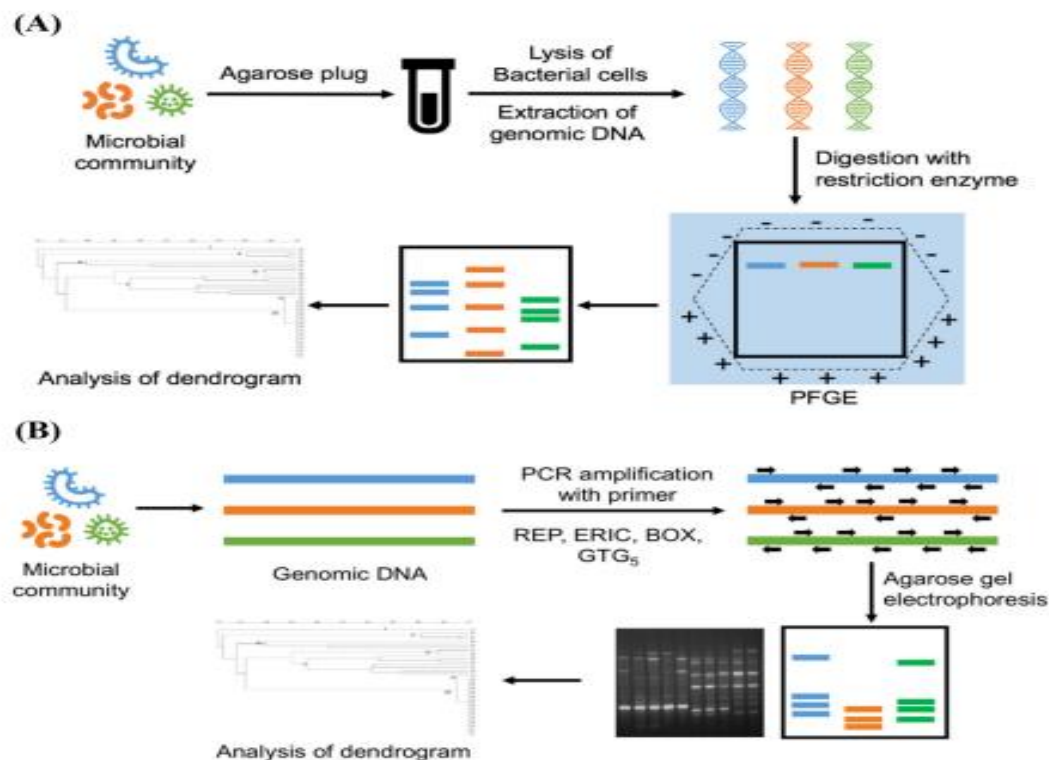


Figure 8. Schematic diagram for pulse field gel electrophoresis (A) and repetitive extragenomic palindromic PCR (B) (Sharma *et al.*, 2020b).

2.5. Technological properties of LAB

LAB are fastidious organisms that require rich and complex nutrients for growth, including carbohydrates, amino acids such as L-glutamic acid, L-isoleucine, L-leucine, L-

tyrosine, and L-tryptophan, as well as vitamins and minerals. Some strains also require special nutrients, such as whey. In addition, LAB can degrade many carbohydrates and related compounds through various metabolic pathways (De Vuyst & Vandamme, 1994).

2.5.1. Acidifying potential

Acidifying power is an important technological property for selecting probiotic strains. During LAB metabolism, several metabolites are produced, mainly lactic acid, the major end-product of carbohydrate fermentation (Figure 9). Lactic acid plays a key role in food technology, particularly in the dairy industry, where it contributes to the fermentation and coagulation of milk. In addition, LAB help preserve fermented foods during production and storage by creating acidic conditions that lower pH and inhibit spoilage microorganisms. In general, the majority of LAB, including species of the genera *Lactobacillus*, *Lactococcus*, and *Leuconostoc*, produce lactic acid as a primary metabolic end product. Lactic acid is also one of the principal antimicrobial substances produced by LAB, exerting antagonistic effects against pathogenic and spoilage microorganisms (De Vuyst & Vandamme, 1994; Salminen & Wright, 1998).

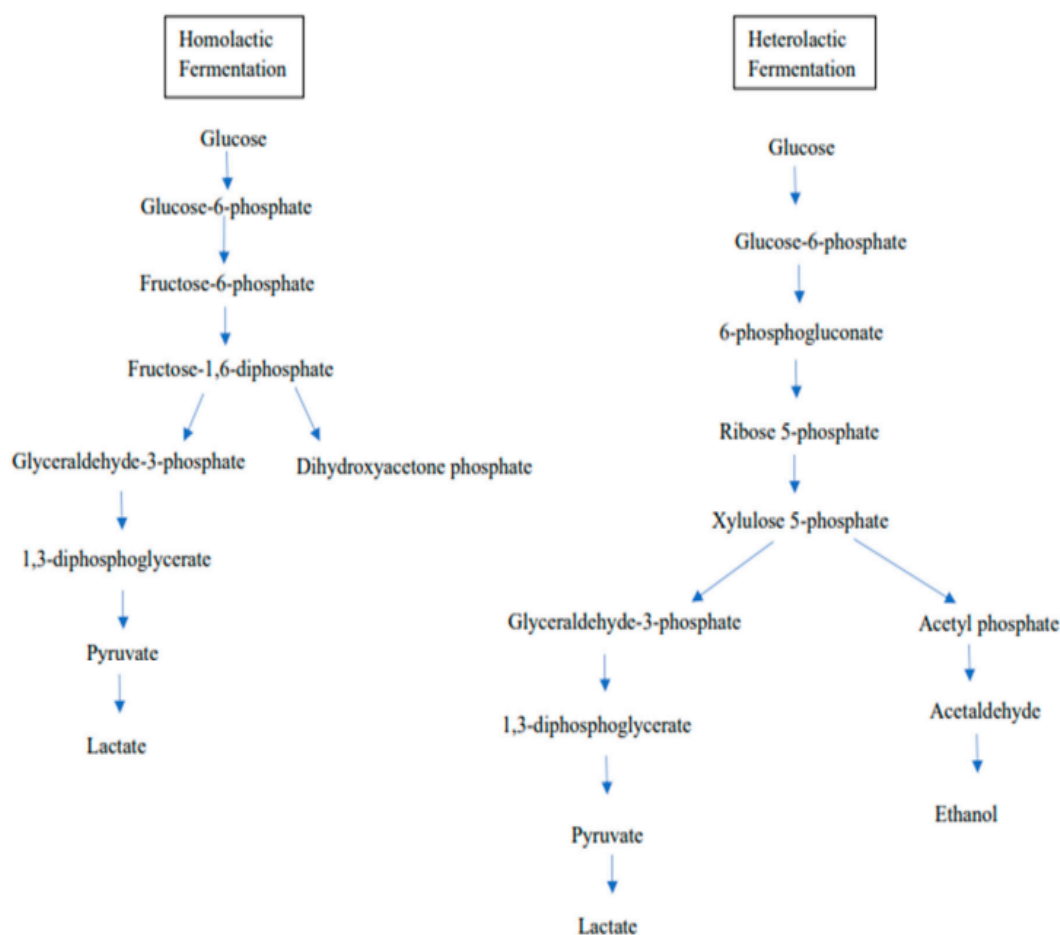


Figure 9. Homolactic and heterolactic fermentative profile of LAB (Wang et al., 2021).

2.5.2. Proteolytic potential

The proteolytic system is a key physiological and biochemical trait of LAB and an important criterion for selecting starter cultures used in fermented dairy products (Savijoki *et al.*, 2006). The proteolytic and peptidolytic enzymes of LAB play a major role in the development of the organoleptic properties of fermented milk products (El-Ghaish *et al.*, 2010). These enzymes enable LAB to hydrolyze milk caseins, releasing peptides and amino acids that serve as nitrogen sources for bacterial growth. LAB are capable of producing amino acids such as tryptophan, lysine, threonine, and methionine, although the level of production depends on the species, subspecies, and strain. Strains of *Pediococcus*, thermophilic *Lactobacillus*, and *Streptococcus* often exhibit strong proteolytic activity and higher amino acid production. Amino acids may be generated through extracellular protein biodegradation, intracellular biosynthetic pathways, or a combination of both (Lim *et al.*, 2019; Kieliszek *et al.*, 2021).

2.5.3. Lipolytic potential

Lipolysis is the hydrolysis of lipids into glycerol and fatty acids through the action of lipolytic enzymes such as lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) (Yerlikaya, 2019). These enzymes catalyze the breakdown of triacylglycerols into diacylglycerols, monoacylglycerols, free fatty acids, and glycerol (Figure 10). Lipases play an important role in the dairy industry by contributing to the development of sensory and nutritional properties, influencing flavor, aroma, and texture in products such as cheese, butter, and yogurt (Oterholm, 1968). Although LAB generally exhibit weak lipolytic activity compared with microorganisms such as *Pseudomonas*, *Aeromonas*, *Acinetobacter*, and *Candida* (Meyers et al., 1996), some LAB strains show measurable lipolytic potential, which varies depending on genus and species. Several methods are available to detect lipase activity in milk and dairy products, including chromatographic, radiometric, turbidometric, colorimetric, fluorimetric, enzymatic, titrimetric, physical, and immunological techniques (Deeth & Touch, 2000; Yerlikaya, 2019; Guan et al., 2020).

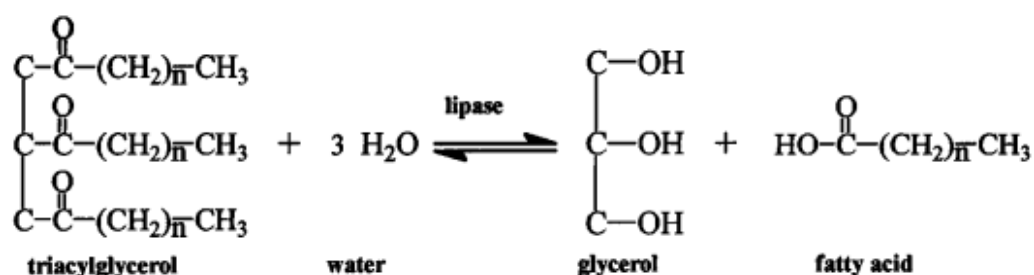


Figure 10. Hydrolytic and synthetic actions of lipase (Thomson et al., 1999).

2.5.4. Aromatic potential

Lactic acid fermentation is a biotechnological process widely used to preserve foods, enhance safety, extend shelf life, and improve sensory characteristics. During fermentation, LAB produce a wide range of aromatic compounds, including α -keto acids, hydroxy acids, aldehydes (e.g., pyruvaldehyde), ketones (e.g., acetone, acetoin, and diacetyl), alcohols, carboxylic acids, and esters (e.g., pentyl acetate). These volatile metabolites, particularly those produced by heterofermentative LAB, play a key role in the flavor development of dairy products such as cheese and butter. Several LAB strains, including *Lactiplantibacillus pentosus*, *Lactobacillus fermentum*, and *Lactobacillus plantarum*, have been evaluated for their capacity to produce these compounds, with

considerable metabolic diversity observed at the strain level. Interestingly, LAB strains with low to moderate proteolytic activity tend to produce higher levels of volatile compounds in milk than highly proteolytic strains. In addition, lipid degradation contributes to aroma formation in fermented dairy products. Consequently, LAB starter cultures with suitable metabolic and enzymatic activities are widely used in the food industry to enhance aroma profiles, with diacetyl and acetoin being among the major flavor compounds whose production varies according to strain metabolism (Lorn *et al.*, 2021; Zareie *et al.*, 2023).

2.4.5. Texturing potential

In the dairy industry, LAB influence the texture of fermented milk through the production of exopolysaccharides (EPS), which is an important technological property in dairy processing. A wide range of LAB species are capable of producing EPS, including *Lactococcus lactis*, *Streptococcus thermophilus*, *Lactilactobacillus*, *Lactocaseibacillus*, *Lentilactobacillus*, *Leuconostoc*, *Limosilactobacillus*, and *Pediococcus*. The texturing potential of EPS is generally evaluated using rheometers or texture analyzers. EPS production and its impact on texture depend on both the bacterial strain and processing conditions. Because of their physicochemical properties, EPS-producing LAB are often used as natural alternatives to commercial food additives, contributing to improved sensory attributes, increased viscosity, and reduced syneresis in fermented products (Poulsen *et al.*, 2019).

Several methods are used to detect and characterize EPS production, such as carbohydrate determination, cell sedimentation in semi-liquid agar, observation of slimy or mucoid colonies on solid media, viscosity assessment in culture broth (e.g., ropiness testing), colorimetric assays including the phenol–sulfuric acid method, high-performance liquid chromatography (HPLC), and gas chromatography (GC). The composition and production of EPS are illustrated in Figure 11 (Korcz&Varga, 2021).

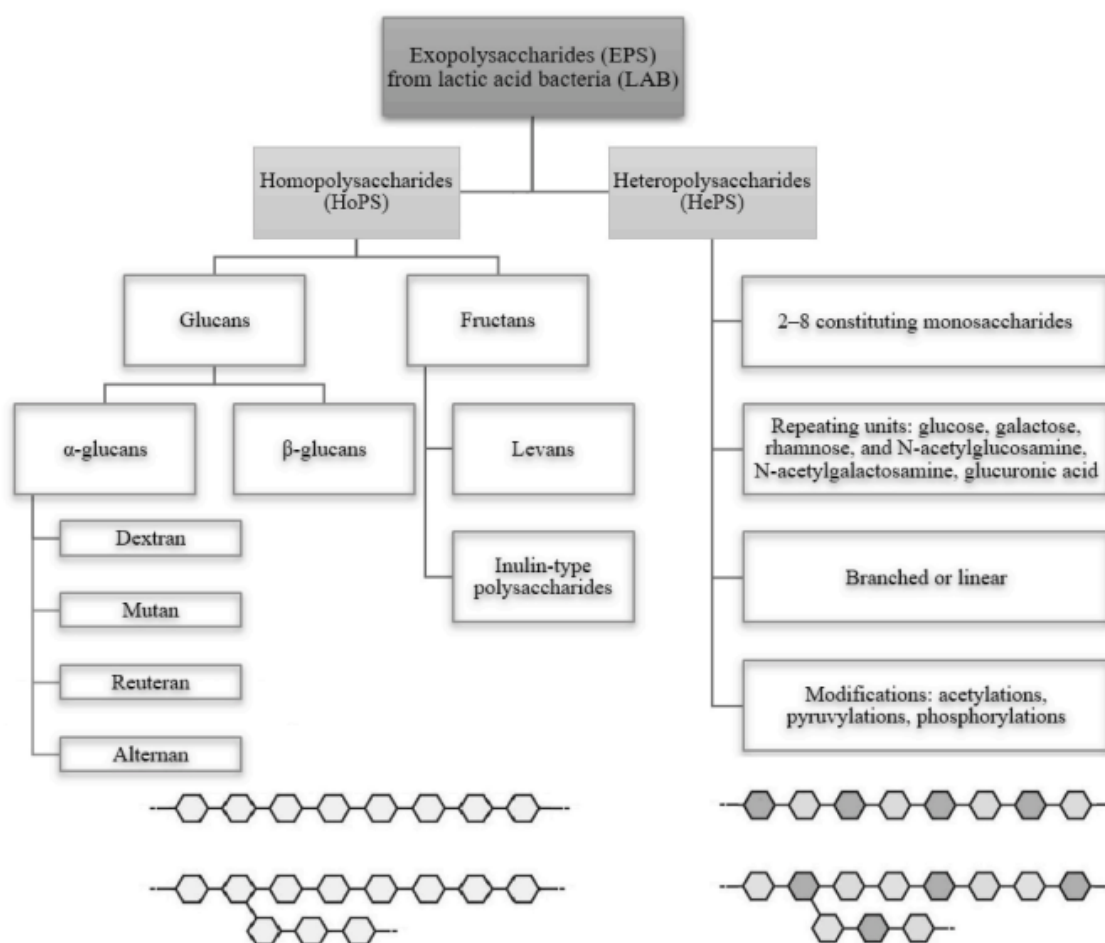


Figure 11. Characterization and classification of exopolysaccharides produced by LAB (Korcz&Varga, 2021).

2.5.6. Antimicrobial potential

Antimicrobial activity is one of the most extensively studied functional properties of LAB, which play a crucial role in food preservation and human health due to their ability to produce a wide range of antimicrobial compounds, including bacteriocins, organic acids (such as lactic and acetic acids), hydrogen peroxide, ethanol, diacetyl, reuterin, acetaldehyde, acetoin, and reutericyclin. These metabolites exert antagonistic effects against numerous food spoilage microorganisms and foodborne pathogens (Reis *et al.*, 2012; De Vuyst&Vandamme, 1994).

In addition, the cell-free supernatant (CFS) of LAB has been shown to inhibit harmful microorganisms through the action of these bioactive metabolites. LAB species, particularly those belonging to the genera *Lactobacillus*, *Enterococcus*, and *Streptococcus*, exhibit strong antimicrobial activity against pathogenic bacteria. Consequently, these inhibitory substances have significant potential for use in the prevention and treatment of infectious

diseases and represent important criteria for the selection of probiotic strains as natural antagonists of pathogenic microorganisms (Girma&Aemiro, 2021; Keeratikunakorn *et al.*, 2023).

2.6. Interactions between LAB and other microorganisms

The microbial ecosystem comprises various types of interactions, including commensalism, predation, parasitism, competition, synergism, and inhibition, particularly within food environments. In the food industry, both heterofermentative and homofermentative LAB are widely used due to their specific properties and cultivation requirements for the production of sour cream, butter, yogurt, fermented beverages, and cheese. Food systems often contain mixed cultures composed of diverse microorganisms that interact with one another, enhancing metabolic activities and contributing to improved product safety and quality. Microbial communities include multiple species and strains, leading to intense competition for limited nutrients within a given niche. This competition occurs mainly in two forms: interference competition and exploitative competition. Interference competition involves direct interactions in which microorganisms inhibit or eliminate competitors through mechanisms such as physical displacement, colonization of available niches, or the production of antimicrobial compounds. In contrast, exploitative competition occurs when species compete indirectly for shared resources, with one species utilizing them more efficiently and thereby limiting availability to others. In addition, LAB can inhibit harmful microorganisms, including molds and other mycotoxin-producing fungi, through competitive exclusion mechanisms involving competition for nutrients and binding sites (Ibrahim *et al.*, 2021).

1. Antimicrobial compounds of LAB

The antimicrobial compounds produced by LAB are commonly divided into low-molecular-mass substances (< 1000 Da) and high-molecular-mass substances (> 1000 Da) (Šušaković *et al.*, 2010).

1.1. Low molecular mass substances

1.1.1. Organic acids

Organic acids are natural compounds found in a wide variety of foods, including vegetables, fruits, and fermented products, and are mainly biosynthesized by microorganisms through fermentation processes. Commonly used organic acids include malic acid (MA), lactic acid (LA), butyric acid (BA), acetic acid (AA), and citric acid (CA). Many organic acids are classified as generally recognized as safe (GRAS) under Food and Drug Administration (FDA) regulations. Organic acids exhibit broad-spectrum antimicrobial activity and high food safety, making them widely used as preservatives to inhibit the growth of bacteria, fungi, and yeasts. They play a crucial role in food preservation while having minimal impact on sensory properties. Additionally, organic acids are low-cost and easy to apply, and they are commonly used in animal production as growth promoters as well as for hygiene and sanitation of food-contact equipment. Organic acids are effective antimicrobial agents because they can penetrate microbial cell membranes in their undissociated form. Once inside the cell, they dissociate and disrupt intracellular pH homeostasis, interfere with metabolic processes, and ultimately lead to cell inactivation. This mechanism is particularly effective under acidic conditions, where the proportion of undissociated organic acid molecules is higher compared to highly dissociating inorganic acids at the same pH (Ji *et al.*, 2023; Sorathiya *et al.*, 2025).

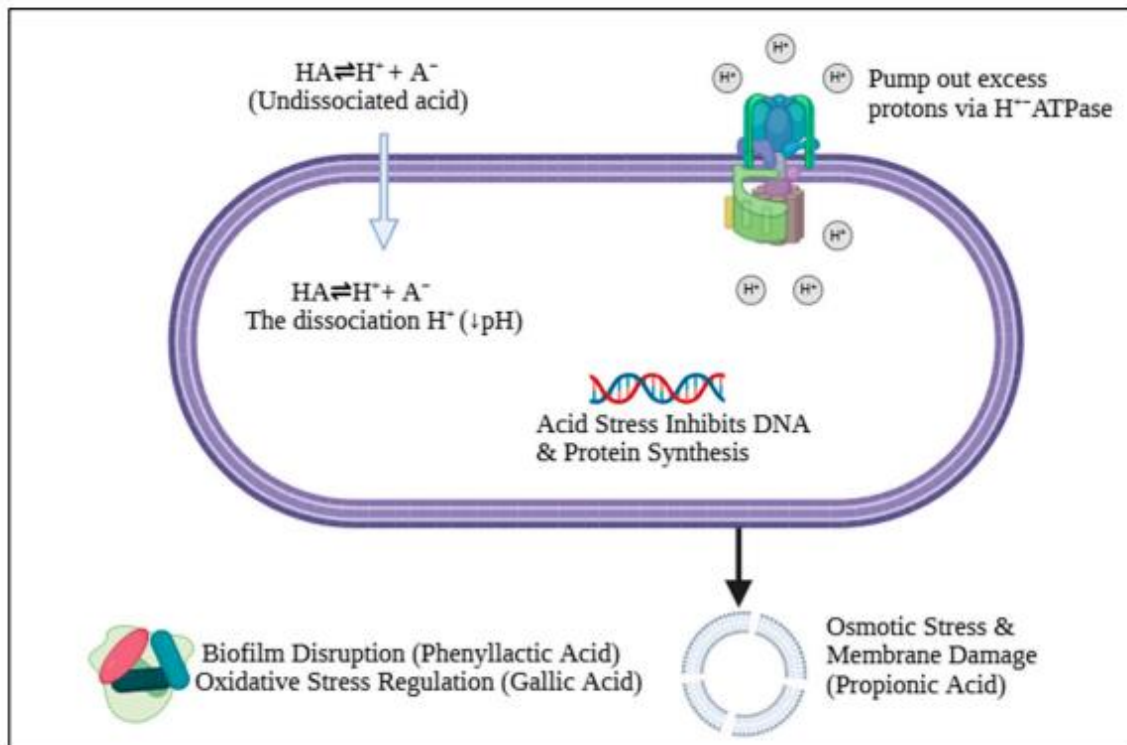


Figure 12. Action mechanism of organic acid on microbial cells. The undissociated acid form diffuses through the microbial membrane when the intracellular pH is higher than the surrounding environment of the cell. Once inside, the intracellular pH of acid causes cell damage (Sorathiya *et al.*, 2025).

1.1.2. Hydrogen peroxide (H₂O₂)

H₂O₂ is one of the major antimicrobial compounds produced by LAB, exhibiting broad-spectrum inhibitory activity against a wide range of microorganisms, including *Candida albicans*, *Escherichia coli*, and *Staphylococcus aureus*. The combined action of lactic acid and H₂O₂ produces a synergistic antimicrobial effect, allowing even low concentrations to effectively inactivate Gram-positive, Gram-negative, and fungal pathogens. In addition to its antimicrobial role, H₂O₂ contributes to dairy preservation by enhancing the aroma and flavor of fermented products. Beyond its natural production by LAB, hydrogen peroxide is widely used industrially as an oxidizing and bleaching agent in the processing of foods such as tea, tripe, herring, beef feet, and dried eggs, and it is classified as a GRAS substance for use in whey and other dairy applications in the United States (AbimbolaEnitan, 2011; Zhang *et al.*, 2019).

1.1.3. Carbon dioxide

CO₂ is typically produced by heterofermentative LAB, including species such as *Leuconostoc mesenteroides* and *Lactobacillus brevis*. Elevated concentrations of CO₂ can exert an inhibitory effect on the growth of various microorganisms, contributing to the preservation of fermented foods (Šušković *et al.*, 2010).

1.1.4. Bacteriocins

Bacteriocins were first discovered in 1925 by Gratia, who observed that *Escherichia coli* V exerted an inhibitory effect against *E. coli* Φ. They are ribosomally synthesized, proteinaceous antimicrobial peptides or proteins produced by a wide range of Gram-positive and Gram-negative bacteria and typically display bactericidal activity against closely related or harmful microorganisms. Bacteriocins produced by LAB are widely used as natural biopreservatives in the food industry due to their heat stability and safety (De Vuyst & Vandamme, 1994; Zhang *et al.*, 2022). Due to their ability to inhibit pathogenic bacteria, bacteriocins are considered promising alternatives to conventional antibiotics. Many probiotic strains produce one or more types of bacteriocins, which contribute to their protective and health-promoting effects. Bacteriocins are classified based on their mechanism of action, the producing microorganisms, and their structural characteristics. In recent years, bacteriocins derived from probiotic strains have attracted increasing interest because of their broad-spectrum antimicrobial activity and associated health benefits (Acedo *et al.*, 2018; Zimina *et al.*, 2020). Bacteriocins can be classified based on the cell wall type of the producing organism as follows:

1.1.4.1. Gram negative bacteriocins

Bacteriocins produced by Gram-negative bacteria are generally classified into four main categories based on their structure and mode of action: microcins, colicins, phage tail-like bacteriocins, and colicin-like bacteriocins.

- ✓ Colicins are synthesized by most *E. coli* strains. They are proteinaceous and sensitive to proteolytic enzymes and high temperatures, which can lead to their degradation. Colicins typically have molecular weights ranging from 30 to 80 kDa.
- ✓ Microcins are produced by members of the Enterobacteriaceae family, including *E. coli* strains. They are low-molecular-weight peptides (~10 kDa) that are resistant to high temperatures, proteases, and extreme pH conditions, and exhibit strong antimicrobial activity

against pathogenic bacteria. Microcin production is typically regulated by gene clusters located on plasmids, and less commonly on chromosomal DNA.

- ✓ Colicin-like bacteriocins have structural and functional properties similar to colicins. They are produced by certain *E. coli* strains and can inhibit or kill pathogenic bacteria.
- ✓ Phage tail-like bacteriocins, also called tailocins, have protein structures ranging from 20 to 100 kDa. These structures are composed of multiple polypeptides and resemble the tail modules of bacteriophages. Tailocins are encoded by gene clusters larger than 40 kbp within bacterial genomes.

1.1.4.2. Gram positive bacteriocins

Gram-positive bacteria, such as LAB, are capable of synthesizing bacteriocins, although the biosynthesis may vary between strains. Bacteriocins produced by Gram-positive bacteria have been classified into four main classes:

- ✓ Class I bacteriocins, known as lantibiotics, are small, heat-stable peptides that contain unusual amino acids such as lanthionine and β -methyllanthionine. Based on their chemical structures and modes of action, they are subdivided into two types: Type A, which includes nisin and has a net positive charge, and Type B, such as mersacidin, which has a negative or no net charge. Class I bacteriocins exhibit antibacterial activity against a wide range of Gram-positive bacteria but typically do not inhibit Gram-negative bacteria. The gene clusters required for their production are usually encoded on plasmids, although they can occasionally be located on chromosomes. Nisin genes are often found on transposons (Lee & Kim, 2011; Suskovic *et al.*, 2010).
- ✓ Class II bacteriocins are small (<60 amino acids), heat-stable peptides that do not contain lanthionine. They represent the best-characterized and most widely distributed group of bacteriocins among food-associated LAB. Class II bacteriocins are further divided into three subgroups:
 - Class IIa bacteriocins, also known as pediocin-like peptides, have an N-terminal consensus sequence Tyr-Gly-Asn-Gly-Val-Xaa-Cys. They exhibit strong antimicrobial activity against foodborne pathogens, particularly *Listeria monocytogenes*, and are widely used as natural biopreservatives in food products.
 - Class IIb bacteriocins are characterized by their requirement of two distinct peptides to exert antimicrobial activity.

- Class IIc and Class IIIc bacteriocins include the remaining peptides of these classes, such as Sec-dependent bacteriocins.
- ✓ Class III bacteriocins, also known as bacteriolysins or non-bacteriocin lytic proteins, are large (>30 kDa), heat-labile antimicrobial proteins. This type of bacteriocin is less well-characterized compared to other classes. Bacteriolysins exert their antimicrobial activity by lysing sensitive cells through catalysis of cell-wall hydrolysis.

1. 2. Enterocin-encoding genes

Enterococci are ubiquitous microorganisms found in a variety of habitats, including fermented dairy products such as Lben, Rayeb, and spontaneously fermented raw-milk cheeses. They play an important role in food and clinical microbiology due to their ability to tolerate high NaCl concentrations, extreme pH values, and variable temperatures. Enterococcus species contribute to the production of fermented foods by providing technological properties and distinctive organoleptic characteristics through diverse enzymatic activities. Additionally, they exhibit antagonistic activity against pathogenic bacteria via the production of hydrogen peroxide, organic acids, and bacteriocins, thereby enhancing the microbiological quality and shelf life of end-products. Some strains are also promising as starter cultures or probiotics due to functional properties such as the absence of antibiotic resistance genes ARGs, tolerance to bile salts, and the ability to adhere to intestinal epithelial cells (Benmouna et al., 2024a; Garmasheva&Oleschenko, 2023).

Several Enterococcus strains produce bacteriocins, known as enterocins, which are of particular interest for food applications. Enterocins are typically screened using PCR-based methods, targeting genes such as entQ, entA, entB, entP, ent31 (bac31), entL50A/B, entX, ent1071A/B, entAS-48, entL, hirJM79, and entSE-K4 (Benmouna *et al.*, 2024b). Some strains carry multiple enterocin gene clusters, although their expression is regulated independently via distinct pathways (Henning *et al.*, 2015).

1.2.1. Enterocin AS-48 (*EntAS-48*) gene

EntAS-48 is produced by *E. faecalis* and belongs to the class IIc cyclic bacteriocins. It can be identified in strains isolated from both clinical sources and foods such as traditional cheeses and milk. *EntAS-48* is composed of 70 amino acid residues and contains neither modified residues nor disulfide bonds. The mature peptide has a molecular mass of 7.14

kDa and an isoelectric point of 10.09. Its structural gene encodes a 105-amino-acid prepeptide that becomes active following the removal of a 35-amino-acid signal sequence. Structurally, EntAS-48 adopts a globular conformation composed of five α -helices surrounding a compact hydrophobic core. Genetically, the *entAS-48* gene cluster is encoded on a pheromone-responsive plasmid and comprises ten genes, including those encoding the structural peptide, the immunity protein, and two ATP-binding cassette (ABC) transporters. This gene cluster has been successfully cloned into several lactic acid bacteria belonging to the genera *Lactobacillus*, *Enterococcus*, and *Lactococcus* (Grande Burgos *et al.*, 2014a).

EntAS-48 was first identified as a peptide antibiotic because of its broad antibacterial spectrum and potential medical applications. It has become a model molecule for understanding how proteins and peptides can evolve to adopt unique structural features that confer exceptional stability and potent antibacterial activity. *EntAS-48* exhibits activity against *Clostridium* species, it is effective also against proliferation of rope-forming *B. licheniformis* and *B. subtilis*. Currently, *entAS-48* is considered one of the most extensively studied bacteriocins. It shows remarkable stability across a wide range of temperatures and pH values, making it an excellent candidate for various food applications (Silva *et al.*, 2018).

Furthermore, *entAS-48* can be obtained from liquid cultures of both exponentially growing and stationary-phase cells, indicating that its production is associated with primary cell metabolism. It can also be produced using inexpensive food by-products such as whey permeate, opening the possibility for industrial-scale production of bacteriocin preparations suitable for use as food additives. *EntAS-48* is degraded by intestinal proteases such as pepsin and trypsin, which may reduce its potential impact on the gut microbiota when ingested with food; however, it is resistant to aminopeptidases and carboxypeptidases. Notably, *entAS-48* has demonstrated significant synergistic effects when combined with other bacteriocins, such as nisin (Grande Burgos *et al.*, 2014b).

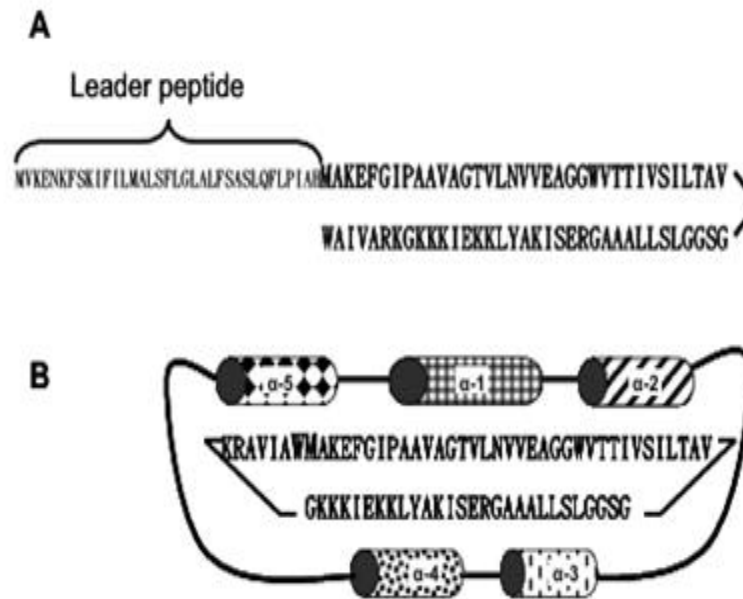


Figure13. Processing of *entAS-48* structural gene product (A) by head-to-tail circularization; and α -helix arrangement of the mature circular peptide (B) (Grande Burgos et al., 2014).

1.2.2. Enterocin 1071A/B (*ent 1071A/B*) gene

Enterocin 1071A/B belongs to the class of two-peptide bacteriocins and is composed of the peptides *Ent1071A* and *Ent1071B*. These peptides were first isolated and characterized from *E. faecalis* BFE 1071, a strain obtained from fecal samples of a minipig. The *ent1071A* and *ent1071B* genes are typically organized in a single operon and may be located either on the chromosome or on plasmids. In *E. faecalis* BFE 1071, these genes are carried on the 50-kbp pEF1071 plasmid. Both *ent1071A* and *ent1071B* are synthesized as prepeptides containing a double-glycine-type leader peptide (Balla et al., 2000a).

Ent1071B from strain BFE 1071 consists of 27 amino acids, whereas *ent1071B* from strain FAIR-E 309 contains 24 amino acids. The molecular masses of *ent1071A* and *ent1071B* produced by strain FAIR-E 309 are 4,284.66 Da and 3,896.15 Da, respectively. The amino acid sequences of *ent1071A* from strains BFE 1071 and FAIR-E 309 are identical and include a lysine at position 38 and an asparagine at position 29. In terms of technological applications, *ent1071* shows considerable potential as a starter culture component in the production of

traditional cheeses, where it can provide protection against pathogenic bacteria such as *L. monocytogenes* (Balla *et al.*, 2000b; Franz *et al.*, 2002a).

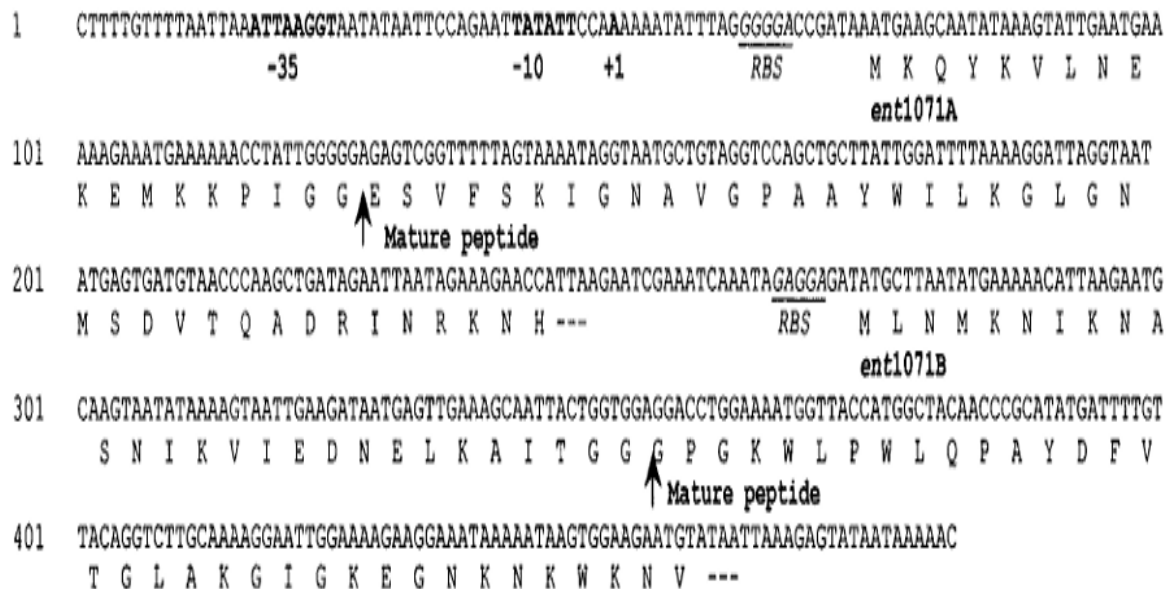


Figure 14. Nucleotide sequence of the regions encoding *ent1071A* and *ent1071B* of *E. faecalis* BFE 1071 and the deduced amino acid sequences (Balla *et al.*, 2000).

1.2.3. Enterocin L50 (*EntL50*) gene

It is produced by *E. Faecium*L50, a strain capable of synthesizing multiple bacteriocins, including *entP*, which belongs to subclass IIa, and *entQ*, a subclass IIc bacteriocin. *EntL50* is part of the large group of antimicrobial agents known as class II bacteriocins, originally described as pediocin-like L50 peptides. Class II bacteriocins are typically small, cationic, hydrophobic, heat-stable, and unmodified peptides with antimicrobial activity. They may consist of a single polypeptide chain or require two complementary peptides for full activity. Additionally, *entL50* present several characteristics that differentiate it from typical class II bacteriocins. This gene can exhibit a broad antimicrobial spectrum against many Gram-positive bacteria, whereas most class II bacteriocins target a narrower range of organisms. Unlike typical class II bacteriocins, which are synthesized as inactive precursors to protect the producer cell, *entL50* is plasmid-encoded and its full activity depends on the combined action of the peptides *entL50A* and *entL50B* (Cintas *et al.*, 1998). Furthermore, the production of bacteriocins by *E. faecium* L50 is

temperature-dependent; in particular, entL50 is synthesized at temperatures ranging from 16 to 32 °C (Criado *et al.*, 2006a).

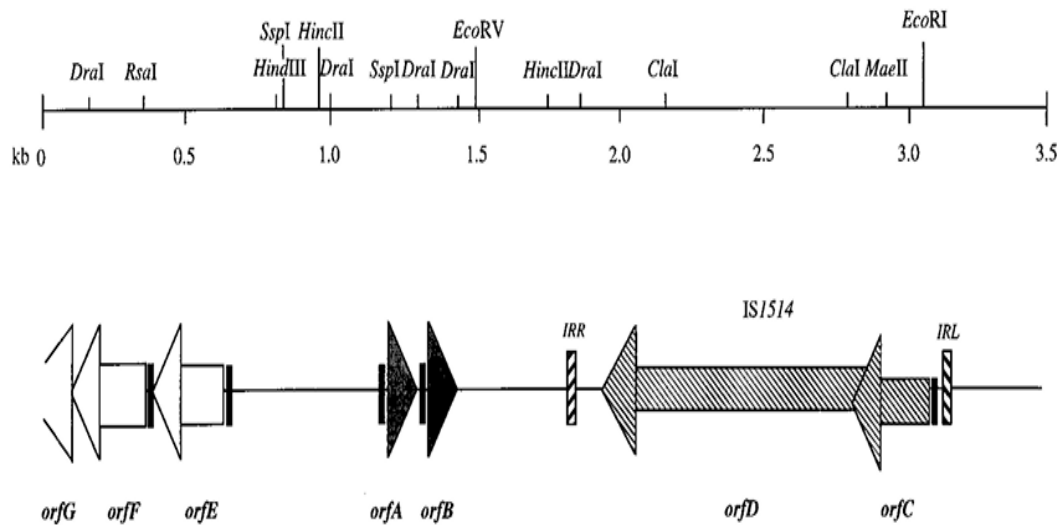


Figure 15. Genetic organization and partial restriction map of the 3.5-kb pCIZ1 fragment containing the enterocin L50A and enterocin L50B structural genes (orfA and orfB) and surrounding ORFs (Cintas *et al.*, 1998).

1.2.4. EnterocinQ (EntQ) gene

EntQ is produced by *E. faecium* L50, a strain originally isolated from a Spanish dry-fermented sausage. This strain carries two plasmids, pCIZ2 (approximately 7.4 kb) and pCIZ1 (approximately 50 kb). Both *entL50* and *entQ* are known to encode leaderless bacteriocins, which are secreted by a mechanism that has not yet been identified. Interestingly, *entQ* is produced over a temperature range of approximately 16 to 47°C. The production of many bacteriocins confers to *E. faecium* L50 a broad spectrum of antimicrobial activity towards a wide range of microorganisms such as *Clostridium perfringens*, *Listeria monocytogenes*, and *Clostridium botulinum*, and activity against animal and human clinical pathogens, including *Streptococcus parasanguis*, *Streptococcus pneumoniae*, *Streptococcus oralis*, *Streptococcus mitis*, and *Streptococcus agalactiae* (Criado *et al.*, 2006b).

1.3. Biosafety of LAB

1.3.1. Antibiotic resistance genes

Recently, a wide range of antibiotics has been frequently detected in groundwater,

surface water, sediments, and soil, making the control of antibiotic residues critically important across different fields. Based on their action mechanism and chemical structure, antibiotics can be classified into many groups: aminoglycosides (AGs), tetracyclines (TCs), β -lactams, sulfonamides (SAs), quinolones (QNs), and macrolides (MLs). ARGs are DNA segments that allow bacteria to withstand the effects of antibiotics. These genes can be acquired through horizontal gene transfer or mutations, processes during which bacteria exchange genetic material, or they may be naturally present in certain bacteria (Wu *et al.*, 2025a).

ARGs complicate the treatment of bacterial infections by enabling bacteria to survive antibiotic therapies that would normally eliminate them, leading to prolonged and persistent infections. Moreover, ARGs can be transferred between different bacterial species, including those that have not previously been exposed to antibiotics, thereby accelerating the spread of resistance and potentially leading to outbreaks of antibiotic-resistant infections (Donkor *et al.*, 2025).

Based on the classes of antibiotics to which they confer resistance, ARGs can be categorized into several groups, including sulfonamides (sul), tetracyclines (tet), β -lactams (bla), aminoglycosides (aac), macrolides (erm), colistin (mcr), fluoroquinolones (fca), multidrug resistance genes (mdr), and vancomycin resistance genes (van). Numerous ARGs have been widely detected in livestock manure worldwide, highlighting their environmental dissemination. Bacterial resistance to antibiotics can be either intrinsic or acquired. Intrinsic resistance refers to the inherent ability of certain bacteria to resist specific antibiotics due to natural structural or functional characteristics, such as the absence of antibiotic targets or limited membrane permeability. In contrast, acquired resistance occurs when bacteria develop resistance through genetic mutations or by acquiring external resistance genes. Acquired resistance mechanisms include the enzymatic inactivation of antibiotics through chemical modification or hydrolysis, reduction of intracellular antibiotic concentrations via efflux pumps or decreased membrane permeability, and alteration of antibiotic targets through genetic mutations or post-translational modifications (Jian *et al.*, 2021). Accordingly, ARGs arise primarily through two mechanisms: mutations within the bacterial genome and horizontal gene transfer involving external DNA acquisition (Wu *et al.*, 2025b).

Overall, the major mechanisms of antibiotic resistance include genetic mutations, efflux pump upregulation, production of antibiotic-inactivating enzymes, ribosomal protection

proteins, interference with folate synthesis pathways, carbapenemase production, reduced expression of outer membrane porins, and the acquisition of resistance-associated genes (Ugbo *et al.*, 2025).

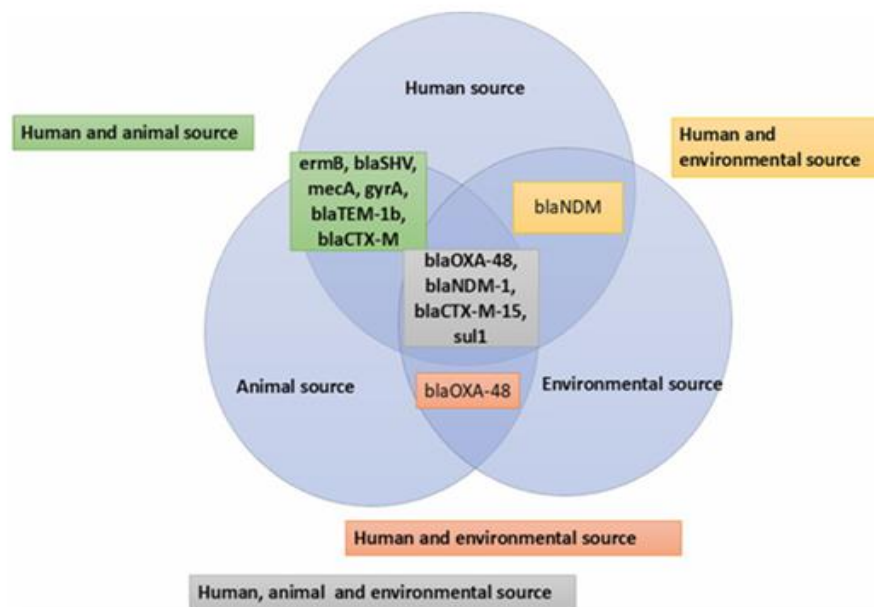


Figure 16. Shared ARGs from human, animal, and environmental (Donkor *et al.*, 2025).

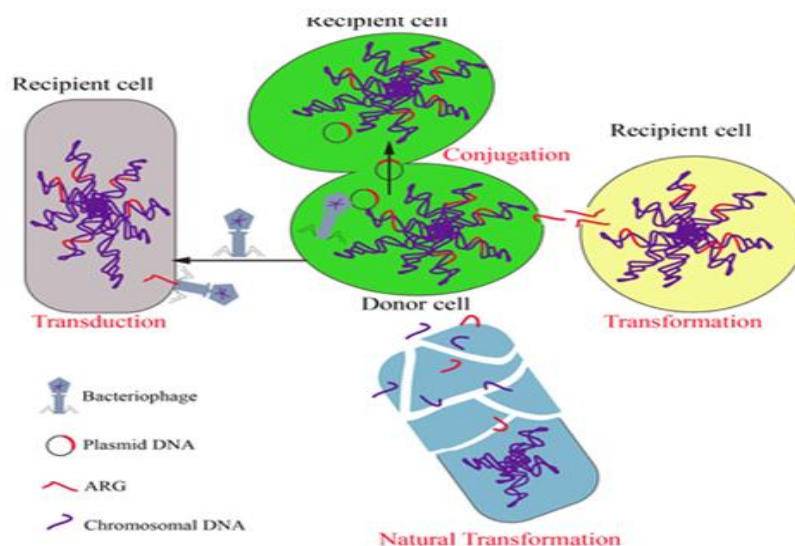


Figure 17. Horizontal transfer of antibiotic resistance genes. Resistance genes are transferred from donor to recipient cells by conjugation, transformation, and transduction (Jian *et al.*, 2021).

1.3.2. Integrons genes

Integrans are genetic elements that play a crucial role in the acquisition and dissemination of antibiotic resistance genes among numerous bacterial species, including those associated with humans. They possess a site-specific recombination system that enables the integration, expression, and exchange of discrete DNA elements known as gene cassettes. Structurally, an integran is characterized by the presence of an integrase gene (*intI*), which mediates recombination, and a primary recombination site called *attI*. Although integrans themselves lack intrinsic mobility, their association with plasmids and transposons allows the horizontal transfer of resistance genes across diverse bacterial species and environments. The gene cassettes carried by integrans are mobile and can be exchanged between different integrans. Based on the sequence similarity of their integrase (*intI*) genes, integrans are classified into several groups, including classes 1, 2, and 3. A class 4 integran, which is associated with chromosomal integrans, has also been described (Bhat *et al.*, 2023).

Class 1 integrans are the most prevalent and are commonly found in Gram-negative bacteria. Structurally, they consist of two conserved regions the 5' conserved segment (5'-CS) and the 3' conserved segment (3'-CS) along with a variable region containing gene cassettes that encode antimicrobial resistance. Class 1 integrans carry a wide variety of resistance gene cassettes, many of which include *aadA* genes that confer resistance to spectinomycin and streptomycin. In contrast, class 2 integrans are less diverse, less frequently detected among Enterobacterales, and are typically associated with transposons of the Tn7 family (Celejewski-Marciniak *et al.*, 2021).

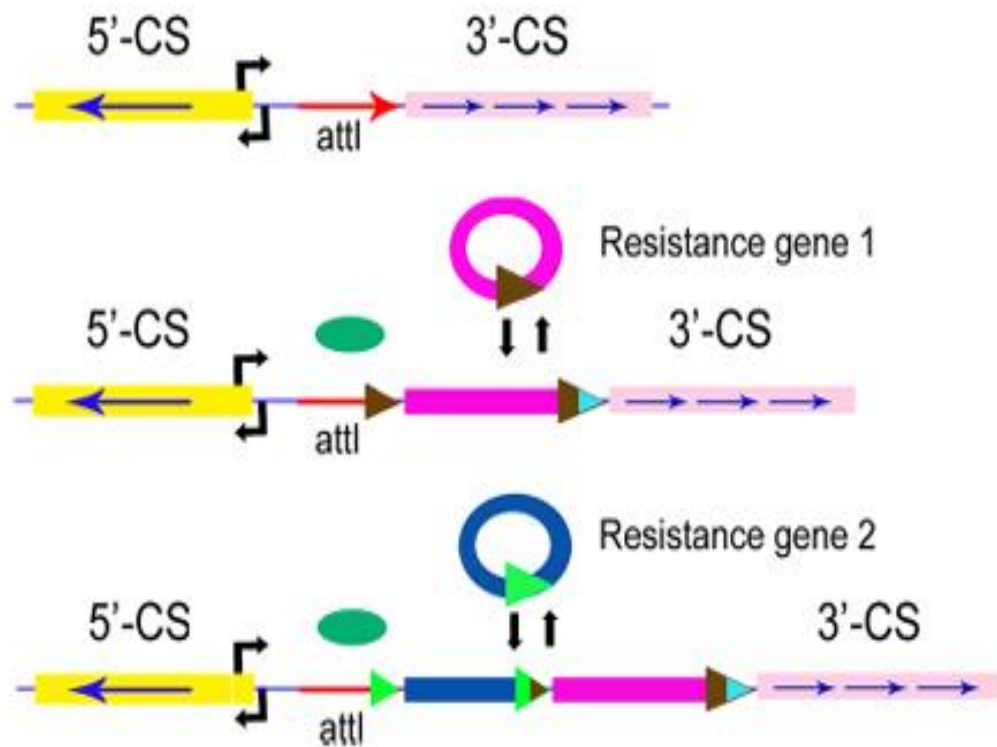


Figure18. Integration and excision of gene cassettes by site-specific recombination (Deng *et al.*, 2015).

1.3.3. Virulence factors

The virulence of microorganisms is generally associated with genes encoding virulence factors, particularly those located in regions known as pathogenicity islands. Virulence factors are surface proteins or other molecules that contribute to a microorganism's ability to cause infectious diseases and pose significant risks to host health. These virulence factors can be divided into two groups: secreted factors and cell surface-associated or adhesion factors (Valencia-Estrada *et al.*, 2024; Iliev *et al.*, 2025). Certain *Enterococcus* species, notably *E.faecalis* and *E. faecium*, display pronounced virulence (Aşgın&Taşkın, 2019a; Heidari *et al.*, 2017).

Although LAB are generally recognized as safe, it remains essential to screen for virulence genes to confirm their safety (Jampaphaeng *et al.*, 2017; Keter *et al.*, 2022). Identified virulence factors include gelatinase (*GeI*), enterococcal surface protein (*Esp*), aggregation substance (*Asa1*), hyaluronidase (*Hyl*), cytolysin (*CylA*), and collagen-binding protein (*Ace*). These factors contribute to colonization, adherence, extracellular enzyme production, evasion of the host immune response, infection severity, and overall

pathogenicity. Some of these genes are chromosomal, while others are acquired via plasmids. Among them, the *esp* gene is one of the most frequently detected (Aşgın&Taşkın, 2019b).

1.3.4. Hemolysis

Hemolysis is the premature destruction of red blood cells (RBCs) and serves as a phenotypic indicator of bacterial virulence. It can result from factors intrinsic or extrinsic to the RBCs (Orf&Cunnington, 2015a). Hemolysis is classified into three types: α -hemolysis, partial or incomplete RBC lysis, as seen in *Streptococcus pneumoniae*; β -hemolysis, complete RBC lysis, as observed in *Staphylococcus aureus*; and γ -hemolysis, in which no hemolysis occurs, as in lactic acid bacteria such as *Lactobacillus*, *Lactococcus*, and *Leuconostoc* species (Kiani *et al.*, 2025).

1.3.5. Biogenic amines

LAB are considered major producers of biogenic amines (BAs) in fermented foods such as vegetables, sausages, and cheeses. During fermentation, these compounds are formed through microbial decarboxylation of amino acids and may exert toxic effects in humans when present at high concentrations. Based on their chemical structure, BAs are classified as aliphatic amines (putrescine, cadaverine, spermidine, and spermine), aromatic amines (tyramine and 2-phenylethylamine), and heterocyclic amines (histamine and tryptamine). The ability of LAB to produce BAs is strain-dependent, showing considerable variability even among strains of the same species. BA formation is influenced by intrinsic and extrinsic factors, including oxygen availability, temperature, pH, carbon sources, amino acid precursors, growth factors, and bacterial growth phase. This capacity is linked to the presence of specific genes or operons encoding amino acid decarboxylases or other pathways involved in BA biosynthesis (Barbieri *et al.*, 2019; Özoğul *et al.*, 2012a).

LAB genera most frequently associated with BA production include *Streptococcus*, *Enterococcus*, *Oenococcus*, *Lactococcus*, *Pediococcus*, and *Lactobacillus*, whereas *Leuconostoc* and *Weissella* are generally considered less active producers. Selecting LAB starter cultures lacking BA-producing pathways is therefore essential for producing high-quality foods with reduced BA levels. However, additional strategies are required to limit BA accumulation, including the use of food microorganisms capable of degrading BAs already formed in the food matrix (Lonvaud-Funel, 2001; Özoğul *et al.*, 2012b).

Materials & Methods

Chapter 1

***First report of enterocin genes in autochthonous
Lacticaseibacillus paracasei and Levilactobacillus brevis
strains isolated from artisanal dairy products made from raw
cow milk***



Research paper

First report of enterocin genes in autochthonous *Lactocaseibacillus paracasei* and *Levilactobacillus brevis* strains isolated from artisanal dairy products made from raw cow milk

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ABSTRACT

Eighteen autochthonous bacteriocin-producing lactic acid bacteria (LAB) isolated from various Algerian artisanal dairy products were identified and characterized using phenotypic and genotypic methods. MALDI-TOF analysis identified 13 isolates (72.22 %) as *Lactocaseibacillus paracasei* subsp. *paracasei* and 5 (27.77 %) as *Levilactobacillus brevis*. The absence of the *Enterococcus*-specific *tuf* gene in all isolates further confirmed the identification results. Most of the isolates exhibited strong antibacterial activity against six pathogenic bacteria, with inhibition zone diameters ranging from 10.67 ± 0.58 mm to 27.00 ± 0.00 mm. PCR screening for twelve enterocin-encoding genes detected the presence of *entAS-48* and *entQ* genes in five and four isolates, respectively. Notably, one *Levilactobacillus brevis* isolate harbored both genes. None of the other enterocin genes were detected in the LAB isolates analyzed. This is the first report of enterocin-encoding genes in non-*Enterococcus* species, highlighting their potential as novel natural food preservatives and emphasizing Algerian artisanal dairy products as a rich source of bacteriocin-producing LAB.

1. Introduction

Artisanal dairy products are widely appreciated by consumers worldwide for their rich nutritional value, functional benefits, and distinctive sensory attributes (Bentahar et al., 2024; Kawęcka & Pasternak, 2022; Rodrigues et al., 2020). Traditional dairy products hold deep social, economic, and cultural importance. Their consumption is rooted in long-standing traditions, closely tied to regional resources and ancestral livestock farming practices that rely on traditional methods and techniques (Gobbetti et al., 2018). Algerian artisanal dairy products may include buttermilk, fresh cream, J'ben, S'men, Lben, Zebda, and Raïb, each providing unique textures and flavors (Leksir et al., 2019).

While deeply rooted in artisanal practices, several of these products have transitioned to industrial production and are now widely available across the country, reflecting their growing popularity and cultural significance (Bousbia et al., 2017; Leksir et al., 2019).

During dairy processing, biochemical transformations are largely influenced by the metabolism of the natural microbiota, with artisanal dairy products serving as a rich source of microorganisms that offer remarkable technological properties (Bouchibane et al., 2023). Among these microbiota, lactic acid bacteria (LAB) are dominant and play a crucial role in the food industry due to their considerable technological and functional properties (Hadeef et al., 2023). Pasteurization of milk is frequently used to eliminate pathogens and extend shelf life. However, it

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also destroys the native microbiota that confers the functional properties of traditional dairy products (Gobbetti et al., 2018). To preserve the native microbiota in their products, many farmers and producers opt to use raw milk in dairy processing (de Medeiros Carvalho et al., 2019). These products continue to be widely accepted and consumed today. In recent decades, artisanal dairy products made from raw milk have gained significant attention from researchers worldwide, as they are an excellent source of novel and autochthonous LAB strains with various technological benefits (Hadeef et al., 2023; Rushdy & Gomaa, 2013). *Lactobacillus* and *Enterococcus* isolates are among the most abundant and commonly recovered LAB in artisanal dairy products (Bouchibane et al., 2023; Silva et al., 2023). In recent years, they have been the focus of extensive research due to their health-promoting properties (Bentahar et al., 2024; Bouchibane et al., 2025).

Antibacterial potential is considered a key and widely studied trait for selecting novel probiotic candidates with broad-spectrum antimicrobial activity (Reis et al., 2012). This antimicrobial activity is primarily attributed to the production of bacteriocins, known as enterocins in *Enterococcus*. Bacteriocins are ribosomally synthesized antimicrobial peptides produced by bacteria that inhibit the growth of pathogenic bacteria (Silva et al., 2020). Bacteriocin-encoding genes are frequently carried on mobile genetic elements such as plasmids, transposons, or integrative conjugative elements, allowing for horizontal genetic transfer among bacterial populations (Fernández et al., 2007).

The transfer of bacteriocin-encoding genes between different genera may lead to the emergence of LAB strains with new traits, ranging from antibiotic resistance to metabolic capabilities, which enhance their ability to adapt to changing environments. Several studies have documented the horizontal gene transfer of bacteriocin-encoding genes between different genera (Criado et al., 2006; Martínez-Bueno et al., 2006). Examining bacteria that harbor naturally acquired bacteriocin-encoding genes, often from different genera, allows for the identification of novel LAB strains with enhanced antibacterial potential. In this regard, this study aimed to isolate, identify, and characterize novel autochthonous bacteriocin-producing LAB strains from various Algerian artisanal dairy products made from raw cow milk, with a particular focus on the characterization of non-genus-specific bacteriocin-encoding genes.

2. Material and methods

2.1. Sample collection

Algerian artisanal dairy products made from raw cow milk, namely Lben, fresh cream, and buttermilk, were selected to carry out this study. These artisanal dairy products were traditionally prepared without the addition of starter cultures. Samples were collected aseptically from several farms located in western Algeria. The samples were transported immediately to the Regional Veterinary Laboratory of Mostaganem, Algeria, in sterile bottles at 4 °C for LAB isolation.

2.2. Isolation and purification of LAB

LAB strains were isolated according to the method previously described by Idoui et al. (2009). For sample preparation, 1 g of Lben was weighed and transferred into 9 mL of TSE buffer. In parallel, 10 g of fresh cream and buttermilk were aseptically mixed with 90 mL of TSE buffer and homogenized. Serial dilutions (from 10^{-1} to 10^{-8}) were then prepared for each sample. After homogenization, 0.1 mL from each dilution was surface-plated onto M17 agar and de Man, Rogosa, and Sharpe (MRS) agar, following the methods described by Terzaghi and Sandine (1975) and De Man et al. (1960). Finally, MRS plates were incubated at 37 °C and 45 °C for 48 h under anaerobic conditions, while M17 plates were incubated aerobically for 72 h. After incubation, individual colonies with distinct morphologies were selected and purified by streaking on fresh MRS and M17 agar plates.

2.3. Identification of LAB

2.3.1. Phenotypic identification

Pre-identification of the isolated strains was performed using the following tests: Gram staining, catalase, oxidase, and motility tests. Pure colonies that were Gram-positive, catalase-negative, oxidase-negative, and non-motile were preserved in MRS and M17 broth containing 30 % glycerol at –20 °C for further analysis.

2.3.2. MALDI-TOF identification

Forty-eight-hour-old colonies grown on Columbia agar with 10 % sheep blood (BioMaxima S.A., Lublin, Poland) in a CO₂-enriched anaerobic jar were identified using the MALDI Biotyper® Sirius System (Bruker, Ettlingen, Germany). This system uses mass spectrometry to identify bacterial strains by comparing the spectra of the analyzed species to reference spectra in its database. The entire procedure was carried out according to the manufacturer's guidelines.

2.3.3. DNA extraction and detection of *Enterococcus*-specific *tuf* gene

Genomic DNA extraction was performed using the boiling method as previously described (Lengliz et al., 2021). To determine whether the strains belonged to the *Enterococcus* genus, the presence of *Enterococcus*-specific *tuf* gene was investigated by PCR using specific primers as previously described (Ke et al., 1999). The primer sequences, expected amplicon size, and PCR conditions are detailed in Table 1.

2.4. Antibacterial potential

Agar well diffusion assay was used to evaluate the antibacterial potential of LAB isolates following the method described by Reuben et al. (2019), with slight modifications. Six bacterial strains were tested in this study, including three Gram-negative bacteria: *Klebsiella pneumoniae* (*K. pneumoniae*) ATCC 10006; *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853; and *Escherichia coli* (*E. coli*) ATCC 25922; and three Gram-positive bacteria: *Enterococcus faecalis* (*E. faecalis*) ATCC 29212; *Enterococcus faecium* (*E. faecium*) ATCC 6057; and *Staphylococcus aureus* (*S. aureus*) ATCC 25923. These pathogenic isolates were first streaked on nutrient agar and incubated at 37 °C for 24 h. The overnight cultures were then spread uniformly on Mueller-Hinton agar plates using sterile cotton swabs. Wells were aseptically punched into the agar, and each well was filled with 100 µL of the LAB cell suspension. Plates were incubated aerobically at 37 °C for 24 h, and the antibacterial activity was determined by measuring the diameter of the inhibition zones around the wells. All experiments were performed in triplicate, and the mean inhibition zone diameter was recorded.

2.5. Detection of enterocin-encoding genes

All LAB isolates were screened for the presence of twelve enterocin-encoding genes using PCR as described previously for *entA*, *entB*, *ent31* (*bac31*), *entP*, *entQ*, *entL50* A/B, *entX*, *ent1071* A/B, *entAS-48* (Lengliz et al., 2021), and *entL*, *entSE-K4* and *hirJM79* (Araújo et al., 2015) (Table 1). The final reaction volume was 25 µL, and the PCR reaction mixture included 1x PCR buffer, 2.5 mM MgCl₂, 200 µM dNTPs, 0.5 µM of each primer, and 3 U of Taq polymerase (Applied Biosystems, Milan, Italy). The primer sequences, expected amplicon sizes, and the amplification conditions for each enterocin gene are provided in Table 1. PCR reactions were carried out either in multiplex or simplex, depending on the primer annealing temperatures and the length of the DNA fragments produced.

2.6. Statistical analysis

In vitro antibacterial activity testing was performed in triplicate, and the antagonistic effect results were expressed as mean ± standard deviation. Statistical analysis of data was carried out using MedCalc

Table 1PCR conditions and primers used for the detection of *Enterococcus*-specific *tuf* and enterocin genes.

Genes	Primers (Sequence 5'-3')	Size (bp)	Amplification Conditions	References
<i>tuf</i>	f: TACTGACAAACCATTCATGATG r: AACTTCGTCACCAACGCGAAC	112	95 °C/5min. 35 cycles: 95 °C/30s, 55 °C/30s, 72 °C/1 min; 72 °C/7min	Ke et al., 1999
<i>entA</i>	f: AAATATTATGGAATGGAGTGTAT r: GCACTTCCTGGAATTGCTC	475	95 °C/5mn. 35 cycles: 94 °C/30s, 50 °C/30s, 72 °C/10s; 72 °C/7mn	Lengliz et al. (2021)
<i>entB</i>	f: GAAAATGATCACAGAATGCCTA r: GTTGCAATTAGAGTATACATTG	195		
<i>ent31</i> (<i>bac31</i>)	f: TATTACGGAATGGTTTATATTG r: TCTAGGAGCCCAAGGGCC	122		
<i>entP</i>	f: CGTAATGGTGTATTATTGTAAT r: ATGTCCCATACCTGCCAAAC	117	95 °C/5mn. 35 cycles: 94 °C/30s, 48 °C/30s, 72 °C/10s; 72 °C/7mn	
<i>entQ</i>	f: CAAGAAATTTTTCCTATGGC r: CTCTTAAAAATGGTATCGCA	95	95 °C/5mn. 35 cycles: 94 °C/30s, 55 °C/30s, 72 °C/10s; 72 °C/7mn	
<i>entL50 A/B</i>	f: GGGAGCAATCGCAAAATTAG r: ATTGCCCATCTCTCTCAAT	150		
<i>entX</i>	f: GTTTCTGTAAAAGAGATGAAAC r: CCTCTTAATCATTAACCATAC	500	95 °C/5mn. 35 cycles: 94 °C/30s, 50 °C/30s, 72 °C/10s; 72 °C/7mn	
<i>ent1071 A/B</i>	f: GGGGAGAGTCGGTTTATTAG r: ATCATATGCGGGTTGTAGCC	273		
<i>entAS-48</i>	f: GAGGAGTTTCATGATTAAAG r: CATATTGTAAATTACCAAGC	185		
<i>entL</i>	f: CGATTCTGTGTAGGAACC r: GTACATCTCCATATACTTTTCC	1770	95 °C/5mn. 35 cycles: 94 °C/1 min, 51 °C/30s, 72 °C/1.5 min; 72 °C/10mn	Araújo et al. (2015)
<i>entSE-K4</i>	f: GCCACGTATTACGGAATGGTGTC r: TTATCTCCACCTATACCACCTAACAC	146	95 °C/5mn. 35 cycles: 94 °C/30s, 53 °C/30s, 72 °C/10s; 72 °C/7mn	
<i>hirJM79</i>	f: ATGAAAAAGAAAGTATTTAAACATTGTGTATTCTAGG r: ATAAGTTAAGCTTGTACTACCTTCTAGGTGCCATGGACC	250	95 °C/5mn. 35 cycles: 94 °C/30s, 61 °C/30s, 72 °C/10s; 72 °C/7mn	

F: forward; R: reverse; bp: base pairs.

software (MedCalc® Statistical Software version 23.1.6, Ostend, Belgium). Kruskal-Wallis and Friedman tests were used for comparing the antibacterial potential of LAB strains and the susceptibility of the tested pathogenic bacteria, respectively. *P* value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Species identification

3.1.1. Phenotypic identification

In this study, a total of eighteen strains were isolated from different Algerian artisanal dairy product samples (Lben, fresh cream, and buttermilk). The isolates were preliminarily identified as presumptive LAB based on their phenotypic characteristics: Gram-positive, catalase-negative, oxidase-negative, non-spore-forming, and non motile

(Table 2). These isolates were also able to grow under both aerobic and anaerobic conditions. The macroscopic characteristics of their colonies showed different forms and colors; colonies grown on MRS and M17 agar were generally small, with some appearing larger, white, circular, and convex in shape. They exhibited a creamy, smooth, and slimy appearance. Raw cow milk is considered a rich source of LAB, constituting a dominant microbial population prior to pasteurization, as reported by several studies documenting their presence within the natural microbiota of raw cow milk (Akpınar & Yerlikaya, 2021; Baruzzi et al., 2011; Kouadri Boudjelthia et al., 2023).

3.1.2. MALDI-TOF identification

MALDI-TOF identification results revealed that, out of the 18 isolated LAB strains, 13 isolates belonged to *Lactocaseibacillus paracasei* subsp. *paracasei* (*L. paracasei*) (72.22 %) and 5 to *Levilactobacillus brevis* (*Lev. brevis*) (27.77 %) (Table 2). *Lactocaseibacillus* and *Levilactobacillus*

Table 2

Phenotypic and MALDI-TOF identification of lactic acid bacteria isolated from Algerian artisanal dairy products.

Isolate code	Sample	Cell shape	Gram reaction	Catalase reaction	Oxydase	Motility	MALDI-TOF identification
S14	Butter milk	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S18	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S21	Fresh cream	Rod	+	–	–	–	<i>Levilactobacillus brevis</i>
S26	Butter milk	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S29	Fresh cream	Rod	+	–	–	–	<i>Levilactobacillus brevis</i>
S37	Butter milk	Rod	+	–	–	–	<i>Levilactobacillus brevis</i>
S38	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S44	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S56	Butter milk	Rod	+	–	–	–	<i>Levilactobacillus brevis</i>
S61	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S71	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S72	Fresh cream	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S95	Lben	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S96	Lben	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S98	Lben	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S103	Lben	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S105	Lben	Rod	+	–	–	–	<i>Lactobacillus paracasei</i> subsp. <i>paracasei</i>
S108	Lben	Rod	+	–	–	–	<i>Levilactobacillus brevis</i>

(–): Positive reaction; (+): Negative reaction.

are newly introduced genera, resulting from the reclassification of *Lactobacillus paracasei* and *Lactobacillus brevis*, which were previously part of the large genus *Lactobacillus* (Zheng et al., 2020). These two species are commonly found in the natural microbiota of various artisanal dairy products (Hussain et al., 2021; Silva et al., 2023). Their dominance in the sampled artisanal dairy products may be due to the production of antimicrobial compounds, such as bacteriocins, which inhibit the growth of competing bacteria. *L. paracasei* and *Lev. brevis* are generally recognized as safe (GRAS) microorganisms for use in food and feed applications, according to the European Food Safety Authority and the United States Food and Drug Administration (EFSA, 2012; Bampidis et al., 2024; FDA, 2004).

3.1.3. Detection of *Enterococcus*-specific *tuf* gene

The identification of the LAB strains as non-*Enterococcus* spp. was further confirmed by screening for the *tuf* gene. The results of this study revealed the absence of this gene in all tested strains, supporting the MALDI-TOF identification results. The *tuf* gene, encoding elongation factor EF-Tu, enables the accurate identification of *Enterococcus* at the genus level and its discrimination from the other genera of Gram-positive bacteria (Ke et al., 1999). Several studies have reported the presence of *Enterococcus* species in artisanal dairy products (Hadeef et al., 2023; Silva et al., 2023). The total absence of this genus in the sampled artisanal dairy products could be attributed to its sensitivity to antibacterial compounds, potentially produced by the two dominant LAB identified in this study.

3.2. Antibacterial potential

Given the crucial role of LAB in inhibiting pathogenic microorganisms and promoting host health, antibacterial activity has become the most widely studied parameter to date (Hussain et al., 2021; Kouadri Boudjelthia et al., 2023). In this study, all the isolated LAB strains exhibited antibacterial activity against the six tested bacteria, with inhibition zone diameters ranging from 10.67 ± 0.58 mm to 27.00 ± 0.00 mm, except for *L. paracasei* S72, which showed antibacterial activity against four of the six tested bacteria (Table 3). Based on inhibition zone diameters, antibacterial potential of LAB was categorized as strong for diameters ≥ 20 mm, moderate for diameters between 10 mm and 20 mm, and weak for diameters ≤ 10 mm (Haghshenas et al., 2015). According to this classification, the majority of LAB strains isolated in this study exhibited strong antibacterial activity against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853 and *E. faecalis* ATCC 29212, and moderate activity against *K. pneumoniae* ATCC 10006, *E. faecium* ATCC 6057 and *S. aureus* ATCC 25923. However, no statistically significant differences were recorded in the susceptibility of Gram-positive and Gram-negative bacteria to LAB isolates, except for *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853, which presented higher sensitivity to *L. paracasei* S26 compared to *K. pneumoniae* ATCC 10006 ($P < 0.01$). Likewise, previous studies have reported that susceptibility to LAB is strain-dependent (Bouchibane et al., 2023; Hussain et al., 2021). Similarly, no statistically significant differences were observed in the antibacterial activity between LAB isolates, except for *Lev. brevis* S29, *L. paracasei* S38 and *L. paracasei* S44. *Lev. brevis* S29 exhibited strong

Table 3
Antibacterial potential of lactic acid bacteria isolated from Algerian artisanal dairy products.

Isolate	Antimicrobial potential (mm)							Enterocin genes
	<i>E. coli</i> ATCC 25922	<i>K. pneumoniae</i> ATCC 10006	<i>P. aeruginosa</i> ATCC 27853	<i>E. faecalis</i> ATCC 29212	<i>E. faecium</i> ATCC 6057	<i>S. aureus</i> ATCC 25923	P value	
<i>L. paracasei</i> S14	$11.33 \pm 0.58^{**}$	17.00 ± 2.00	25.00 ± 0.00	23.67 ± 2.31	19.00 ± 1.00	15.00 ± 0.00	<0.00001	entAS-48
<i>L. paracasei</i> S18	16.67 ± 0.58	17.00 ± 1.00	24.00 ± 0.00	22.33 ± 0.58	16.00 ± 0.00	12.67 ± 0.58	<0.00001	
<i>L. paracasei</i> S26	17.00 ± 1.00	$14.33 \pm 1.15^{**}$	$18.00 \pm 0.00^{**}$	17.67 ± 0.58	15.00 ± 0.00	$18.67 \pm 1.53^{**}$	0.00268	entAS-48
<i>L. paracasei</i> S38	$15.67 \pm 0.58^{**}$	15.67 ± 0.58	26.00 ± 2.00	21.67 ± 1.53	19.00 ± 1.00	$25.00 \pm 0.00^{*}$	<0.00001	entQ
<i>L. paracasei</i> S44	22.00 ± 0.00	18.00 ± 0.00	21.00 ± 0.00	21.00 ± 1.00	15.67 ± 0.58	$25.00 \pm 0.00^{*}$	<0.00001	entQ
<i>L. paracasei</i> S61	18.67 ± 1.53	18.00 ± 1.00	24.67 ± 4.62	22.00 ± 2.00	17.67 ± 0.58	15.67 ± 0.58	0.00021	entAS-48
<i>L. paracasei</i> S71	21.00 ± 0.00	15.67 ± 0.58	21.67 ± 0.58	19.67 ± 0.58	15.33 ± 1.16	19.67 ± 0.58	<0.00001	entQ
<i>L. paracasei</i> S72	20.00 ± 0.00	<6	19.00 ± 1.00	16.67 ± 1.53	<6	15.67 ± 0.58	0.00203	
<i>L. paracasei</i> S95	21.67 ± 0.58	14.67 ± 0.58	19.67 ± 0.58	16.67 ± 0.58	15.00 ± 0.00	11.00 ± 0.00	<0.00001	
<i>L. paracasei</i> S96	17.67 ± 0.58	16.33 ± 0.58	21.67 ± 0.58	22.00 ± 0.00	16.00 ± 0.00	12.00 ± 0.00	<0.00001	
<i>L. paracasei</i> S98	20.00 ± 0.00	18.33 ± 1.53	24.33 ± 0.58	26.00 ± 0.00	19.00 ± 0.00	11.00 ± 1.00	<0.00001	
<i>L. paracasei</i> S103	24.00 ± 0.00	16.00 ± 0.00	23.33 ± 2.08	18.67 ± 1.53	15.00 ± 0.00	10.67 ± 0.58	<0.00001	
<i>L. paracasei</i> S105	17.67 ± 0.58	16.00 ± 1.00	21.33 ± 0.58	22.00 ± 0.00	18.00 ± 2.65	10.67 ± 0.58	<0.00001	
<i>Lev. brevis</i> S21	22.00 ± 0.00	16.33 ± 0.58	22.00 ± 0.00	20.00 ± 1.00	15.00 ± 0.00	$9.67 \pm 0.58^{*}$	0.01036	
<i>Lev. brevis</i> S29	$27.00 \pm 0.00^{**}$	16.33 ± 0.58	22.33 ± 2.52	18.67 ± 0.58	14.00 ± 0.00	11.67 ± 0.58	0.01036	entAS-48, entQ
<i>Lev. brevis</i> S37	21.67 ± 0.58	16.00 ± 0.00	21.00 ± 0.00	19.33 ± 1.16	14.67 ± 0.58	22.00 ± 0.00	<0.00001	entAS-48
<i>Lev. brevis</i> S56	24.00 ± 0.00	15.33 ± 0.58	21.33 ± 0.58	20.00 ± 0.00	14.67 ± 0.58	10.67 ± 0.58	<0.00001	
<i>Lev. brevis</i> S108	17.67 ± 0.58	17.67 ± 1.15	24.00 ± 1.00	23.00 ± 1.00	19.67 ± 0.58	11.67 ± 0.58	<0.00001	
P value	0.000008	0.000420	0.000099	0.000022	0.000072	0.000007		

L. paracasei: *Lactobacillus paracasei* subsp. *paracasei*; *Lev. brevis*: *Levilactobacillus brevis*; *E. coli*: *Escherichia coli*; *K. pneumoniae*: *Klebsiella pneumoniae*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *E. faecalis*: *Enterococcus faecalis*; *E. faecium*: *Enterococcus faecium*; *S. aureus*: *Staphylococcus aureus*. $^{*}/^{**}$ Isolates with the same number of asterisks are significantly different from each other.

antibacterial activity against *E. coli* ATCC 25922, and *L. paracasei* S38 and *L. paracasei* S44 showed significant antibacterial activity against *S. aureus* ATCC 25923 ($P < 0.05$). These findings highlight the broad-spectrum antibacterial potential of the isolated LAB strains. Several studies have recently investigated the antimicrobial activity of LAB strains isolated from various artisanal dairy products and raw cow milk (Bentahar et al., 2024; Bouchibane et al., 2023; Zhang et al., 2022). Recently, Bentahar et al. (2024) investigated the antibacterial activity of three *Lactobacillus* isolates, two *Lev. brevis* and one *Lactiplantibacillus plantarum*, obtained from Dhan, a traditional Algerian cow milk butter, against *E. coli* ATCC 10536, *P. aeruginosa* ATCC 27853, and *S. aureus* ATCC 25923. Their results showed that most isolates exhibited only weak to moderate antibacterial potential. In their study, Bouchibane et al. (2023) showed that certain LAB strains, such as *Limosilactobacillus fermentum*, *Lactiplantibacillus plantarum*, *Lactiplantibacillus pentosus*, and *E. faecium*, isolated from Algerian artisanal dairy products, exhibited inhibitory effects against *E. coli* ATCC 25922. They also reported that strains belonging to the genus *Enterococcus* produced smaller inhibition halos compared to those of the genus *Lactobacillus*. In another work, Rushdy and Gomaa (2013) demonstrated that the antimicrobial compound produced by one *Lev. brevis* strain, isolated from traditional Egyptian cheese, exhibited inhibitory activity against both Gram-positive and Gram-negative bacteria. Compared to the findings of these previous studies, the results of the current study showed much stronger antimicrobial activity of LAB, highlighting the substantial antibacterial potential of LAB strains isolated from Algerian artisanal dairy products made from raw cow milk. In fact, raw cow milk has been reported to be a rich source of indigenous LAB with notable probiotic potential (Zhang et al., 2022).

3.3. Detection enterocin-encoding genes

Molecular characterization revealed the presence of bacteriocin-encoding genes in eight isolates (44.44 %) (Table 3). The enterocin genes *entAS-48* and *entQ* were detected in five and four isolates, respectively. However, the genes *entA*, *entB*, *ent31* (*bac31*), *entP*, *entL50 A/B*, *entX*, *ent1071 A/B*, *entL*, *entSE-K4* and *hirJM79* were not detected in any of the isolates. The two detected enterocin genes encode antimicrobial peptides that are commonly found in *Enterococcus* species, especially *E. faecalis* and *E. faecium* (Cintas et al., 2000; Criado et al., 2006; Grande Burgos et al., 2014). It has been reported that enterocin AS-48 exhibits strong antimicrobial activity against various Gram-positive bacteria, including *S. aureus*, *Bacillus cereus*, and *Listeria monocytogenes* (Ananou et al., 2010; Samyn et al., 1994). Its mechanism of action involves the permeabilization of the bacterial cytoplasmic membrane via pore formation, disrupting the proton motive force and thereby inducing cell death (Maqueda et al., 2004). Due to its significant stability against extreme pH, heat, and denaturants, enterocin AS-48 has been widely employed as a bio-preservative in food systems (Grande Burgos et al., 2014). Similarly, enterocin Q has demonstrated antimicrobial activity against certain Gram-positive bacteria, including *E. faecium* (Cintas et al., 2000). The detection of *Enterococcus*-specific bacteriocin genes in this study in *L. paracasei* and *Lev. brevis* may be explained by their horizontal transfer, potentially mediated by mobile genetic elements. Indeed, it has been reported that these genes reside on conjugative plasmids: pMB2 for *entAS-48* and pCIZ2 for *entQ* (Criado et al., 2006; Martínez-Bueno et al., 1994). Furthermore, several studies have investigated the horizontal transferability of these two enterocin genes across genera (Criado et al., 2006; Fernández et al., 2007; Martínez-Bueno et al., 2006). Fernández et al. (2007) successfully cloned the *entAS-48* gene cluster into several strains of LAB. Likewise, Criado et al. (2006) reported that the *entQ* gene could be heterologously expressed in *Lactobacillus sakei*. Additionally, *Lactobacillus* isolates are frequently found in various artisanal dairy products, where they often share their niche with *Enterococcus* strains (Bouchibane et al., 2023; Hadeef et al., 2023; Silva et al., 2023). The dense microbial communities and intense

competitive pressures in these products may create a selective environment conducive to the horizontal transfer of plasmids carrying bacteriocin genes. The presence of these two bacteriocin genes in *L. paracasei* and *Lev. brevis* isolates may also be explained by the presence of homologous genes, as no study has yet investigated their occurrence in such isolates. The natural occurrence of *entAS-48* and *entQ* enterocin genes in *L. paracasei* and *Lev. brevis*, which are widely recognized as GRAS (Bampidis et al., 2024; EFSA, 2012; FDA, 2004), represents a significant and novel finding, highlighting the first report of these enterocin genes outside the genus *Enterococcus*, that has not yet achieved GRAS status (Huys et al., 2013) due to concerns over opportunistic pathogenicity and antibiotic resistance.

This study also reported that the *Lev. brevis* S29 isolate harbored both *entAS-48* and *entQ* genes. This isolate exhibited greater antibacterial activity against *E. coli* in comparison to the other LAB isolates, suggesting that these genes may be functional. However, the differences were not statistically significant except in comparison with *L. paracasei* S14 and *L. paracasei* S38 ($P < 0.001$), which harbored the *entAS-48* and *entQ* genes, respectively. This enhanced activity may be attributed to the synergistic effect between the two enterocins. Multiple bacteriocin production by LAB has been documented in several studies (Lengliz et al., 2021; Perez et al., 2012). The presence of both *entAS-48* and *entQ* genes in the same non-*Enterococcus* spp. strains may be explained by the horizontal acquisition of mobile genetic elements carrying both genes in a physically linked arrangement.

The presence of the *entAS-48* and *entQ* genes may enhance the competitive fitness of these LAB strains by equipping them with potent antimicrobial mechanisms against pathogens. This could partially explain the substantial antibacterial activity of the LAB strains isolated in this study. However, all LAB strains isolated in this study generally exhibited strong antibacterial effects against most of the tested pathogenic bacteria, and no statistically significant differences were observed between enterocin-producing and non-enterocin-producing strains. The significant antibacterial activity of the non-enterocin-producing LAB isolates may be due to the presence of other bacteriocin-encoding genes that were not investigated in this study. The expression and functionality of the *entAS-48* and *entQ* genes in *Lactobacillus* isolates, along with the genetic elements involved in horizontal gene transfer were also not analyzed. This limits our understanding of their role in the observed antibacterial activity and the mechanisms underlying their acquisition.

4. Conclusion

This finding reports, for the first time, the natural occurrence of enterocin-encoding genes in *L. paracasei* and *Lev. brevis* isolates and highlights Algerian artisanal dairy products as a rich reservoir of autochthonous LAB isolates with significant antibacterial potential, suggesting their potential as novel natural food preservatives. However, this study should be continued to investigate the origin, expression, and functionality of the *entAS-48* and *entQ* genes in *Lactobacillus* isolates. Further studies should also focus on identifying atypical or exogenous genes within GRAS microorganisms in order to trace their evolution, ensure their continued safety, and uncover novel traits that may enhance their technological or therapeutic potential.

CRedit authorship contribution statement

Roumaissaa Belkacem: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Qada Benameur:** Writing – review & editing, Writing – original draft, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Smaranda Crăciun:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Data curation. **Sana Lengliz:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Hajer**

Kilani: Writing – review & editing, Validation, Methodology, Formal analysis, Data curation, Conceptualization. **George Cosmin Nădăș:** Writing – review & editing, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Adriana Györke:** Writing – review & editing, Visualization, Validation, Supervision, Software, Investigation, Formal analysis, Data curation, Conceptualization. **Mohamed Salah Abbassi:** Writing – review & editing, Validation, Supervision, Methodology, Investigation, Formal analysis, Data curation, Conceptualization.

Data availability statement

Data is contained within the article.

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.

Data availability

Data will be made available on request.

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Chapter 2

Biotechnological Characterization and Safety Assessment of Lacticaseibacillus paracasei and Levilactobacillus brevis Strains Carrying entAS-48 and entQ Genes

Article

Biotechnological Characterization and Safety Assessment of *Lacticaseibacillus paracasei* and *Levilactobacillus brevis* Strains Carrying *entAS-48* and *entQ* Genes

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Abstract

In our previous work, we reported for the first time the presence of enterocin-encoding genes in novel *Lacticaseibacillus paracasei* (*L. paracasei*) and *Levilactobacillus brevis* (*Lev. brevis*) strains isolated from artisanal dairy products made from raw cow milk. The aim of this study was to isolate enterocin-positive lactic acid bacteria (LAB) from artisanal dairy products and assess their technological characteristics and safety for potential application in food systems. LAB isolates were characterized using phenotypic tests, Matrix-Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) identification, and PCR detection of enterocin genes, followed by evaluation of their physiological and technological properties and a comprehensive safety assessment, including antimicrobial resistance, virulence, biogenic amine, and integron genes. Two strains, *L. paracasei* S2 and *Lev. brevis* S62, carried enterocin genes (*entAS-48* and *entQ*) and exhibited strong acidifying and proteolytic activities, along with antibacterial effects against foodborne pathogens and reference strains. Both isolates tolerated environmental stresses, including low pH, and lacked virulence factors, clinically relevant antibiotic resistance genes, biogenic amine production, and integron elements. These results indicate that the strains are safe, multifunctional, and suitable for developing regionally adapted dairy products, highlighting artisanal dairy products as a valuable source of novel LAB with promising biotechnological applications.

Keywords: biotechnological properties; enterocin genes; *Lacticaseibacillus paracasei*; *Levilactobacillus brevis*; safety



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1. Introduction

Lactic acid bacteria (LAB) represent a diverse group of Gram-positive, non-sporulating, acid-tolerant microorganisms known primarily for their ability to ferment carbohydrates into lactic acid [1]. As bacteriocin producers, probiotics, and starter cultures, LAB have gained significant attention due to their multifaceted applications in food preservation, human and animal health, agriculture, and biotechnology [1–3]. However, the functionality of LAB is highly strain-dependent, and the systematic characterization of indigenous isolates remains critical for identifying candidates with desirable technological and health-related traits [4]. LAB play a significant role in functional food industry due to their biotechnological properties, including acidifying power, proteolytic activity, and antimicrobial potential [5].

LAB strains can be isolated from different niches, including artisanal dairy products [6,7]. Autochthonous LAB strains isolated from traditional dairy products are of particular interest due to their adaptation to specific ecological niches and potential for use in the development of regionally adapted starter or adjunct cultures [8]. Traditional dairy products produced from raw milk without the addition of commercial starter cultures serve as rich reservoirs of diverse and functionally relevant LAB [6,8]. These products are often produced under spontaneous fermentation conditions, promoting the selection of strains with superior resilience and adaptability to environmental stresses such as low pH, elevated osmotic pressure, and temperature fluctuations [9].

Lacticaseibacillus paracasei (*L. paracasei*: formerly *Lactobacillus paracasei*) and *Levilactobacillus brevis* (*Lev. brevis*: previously identified as *Lactobacillus brevis*) are among the most studied LAB due to their broad-spectrum antimicrobial activity and their Generally Recognized as Safe (GRAS) status. Currently, extensive attention is being directed toward the characterization of the technological properties of LAB strains carrying bacteriocin genes, which may further support their diverse application [10,11]. In our previous work, we reported for the first time the presence of enterocin-encoding genes (*entAS-48* and *entQ*) in novel *L. paracasei* and *Lev. brevis* strains isolated from artisanal dairy products made from raw cow milk [12], suggesting the possibility of horizontal gene transfer from *Enterococcus* spp. isolates. Enterocin AS-48 (*entAS-48*) is classified as a circular class IIc bacteriocin, and enterocin Q (*entQ*) belongs to the class IId non-pediocin-like, one-peptide, linear bacteriocins [13]. Their antimicrobial potential and remarkable resistance to harsh environmental conditions, including acidic and alkaline pH levels, high temperatures, and chemical denaturants, have made them a valuable natural preservative in the food industry [14]. The natural occurrence of these enterocins in isolates known for their GRAS status is a relevant finding. However, the biotechnological characteristics of these novel strains have not yet been studied.

Although many LAB species have GRAS status and several are listed under the Qualified Presumption of Safety (QPS) by the European Food Safety Authority, safety traits are highly species- and strain-dependent [15,16]. Several studies have highlighted the importance of conducting comprehensive safety assessments of LAB, particularly those intended for food applications [15,17,18]. These assessments typically focus on the detection of antibiotic resistance [15,19,20], virulence-associated [17,21], and biogenic amine genes [16,18,22]. Previous research has demonstrated considerable variability in these safety attributes among LAB isolated from artisanal dairy products, underscoring the importance of strain-level evaluation [15,16,20]. In this context, the present study was designed to (i) isolate and identify enterocin-positive LAB strains from artisanal dairy products, (ii) evaluate their biochemical and technological properties, and (iii) assess their safety characteristics, including the presence of antibiotic resistance determinants, virulence encoding genes, biogenic amine, and integron genes.

2. Materials and Methods

2.1. Sampling, Isolation and Purification of LAB

Two artisanal dairy product samples, namely fresh cream and butter, were collected from two dairy farms in Mostaganem province, Algeria, for LAB isolation. These products were made from raw cow milk without the addition of any starter. Fresh cream was obtained by incubating raw milk at room temperature to allow the fat to rise, followed by careful manual skimming of the upper cream layer. Butter was produced by churning naturally fermented milk (Rayeb) in a traditional goatskin bag until butter granules formed. During the final stage of churning, warm water (40–50 °C) was added to promote fat coalescence and improve butter yield. The resulting butter was then collected manually or with a strainer and washed several times to remove residual liquid. The isolation of LAB strains was carried out using the previously described method [23]. Briefly, 10 g or mL of each sample was aseptically dissolved in 90 mL of Tris-Saline-EDTA buffer (TSE, Pasteur Institute of Algeria, Algiers, Algeria) buffer and homogenized. Serial dilutions (10^{-1} to 10^{-8}) were prepared for each sample, and 0.1 mL of each dilution was spread onto Man, Rogosa, and Sharpe (MRS) agar and M17 agar (Condalab, Madrid, Spain). The plates were then incubated at 37 °C and 45 °C for 48 h under anaerobic conditions. Single colonies were selected and purified on MRS agar and M17 agar.

2.2. Identification of LAB

2.2.1. Phenotypic Identification

Preliminary identification of the isolated strains was performed following Atfaoui et al. [24], based on Gram staining, cell and colony morphology, and the catalase, oxidase, and motility tests. Colonies that were Gram-positive, catalase- and oxidase-negative, and non-motile were preserved in MRS and M17 broth (Condalab, Madrid, Spain) containing 30% glycerol at −20 °C for subsequent analyses.

2.2.2. MALDI-TOF Identification

The LAB isolates were identified using the MALDI Biotyper[®] Sirius System (Bruker, Ettlingen, Germany), following the previously described method [12]. Briefly, 48-hour-old colonies grown on Columbia agar (BioMaxima S.A, Lublin, Poland) supplemented with 10% sheep blood were transferred onto a polished steel target plate. Each sample was then overlaid with 1 µL of α -cyano-4-hydroxycinnamic acid (HCCA) matrix solution (Bruker, Ettlingen, Germany). After air drying, the samples were analyzed using the MALDI Biotyper[®] Sirius System. The obtained mass spectra were compared with the Bruker reference database for bacterial identification, and the identification scores were interpreted according to the manufacturer's guidelines.

2.2.3. DNA Extraction and Detection of *Enterococcus*-Specific *tuf* Gene

Genomic DNA was extracted using the boiling method, as previously described [25], with slight modifications. Briefly, colonies from overnight culture were suspended in 500 µL of sterile distilled water in 1.5 mL Eppendorf tubes, then boiled at 100 °C for 15 min and immediately chilled at −20 °C for 15 min. After chilling, the tubes were centrifuged at 12,000 rpm for 10 min, and the supernatant was transferred to sterile 1.5 mL Eppendorf tubes. The extracted DNA was stored at −20 °C for further use in PCR assays. To investigate whether the isolates belong to the genus *Enterococcus*, detection of the genus-specific *tuf* gene was carried out using PCR, as previously described [26]. The specific primers and amplification conditions used in this study are described by Belkacem et al. [12].

2.3. Detection of Enterocin-Encoding Genes in LAB Isolates

LAB isolates were screened for the presence of twelve enterocin-encoding genes: *entB*, *entA*, *entL50A/B*, *ent31* (*bac31*), *entQ*, *entP*, *entX*, *entAS-48*, *ent1071A/B*, *entSE-K4*, *entL*, and *hirJM79*. Each PCR reaction was carried out in a final volume of 25 μ L, containing 2.5 mM $MgCl_2$, 1 $\times$ PCR buffer, 0.5 μ M of each primer, 200 μ M of dNTP, and 3 U of Taq DNA polymerase (Applied Biosystems, Milan, Italy). Primers and amplification conditions followed those reported previously [12]. PCR products were separated by electrophoresis on 2% agarose gels stained with ethidium bromide (0.5 μ g/mL) and visualized using a 100 bp DNA ladder (Invitrogen™, Thermo Fisher Scientific, Waltham, MA, USA) as a molecular size marker.

2.4. Physiological Characterization of LAB Isolates

The physiological characteristics of LAB isolates were evaluated following the methods reported by Kavitha et al. [27]. Growth at different temperatures was assessed by incubating cultures in MRS broth at 15 °C for 7 days and at 37 °C and 45 °C for 48 h. Acid tolerance was evaluated by adjusting the pH of MRS broth to 2, 3, 4, and 5, followed by incubation at 37 °C, while salt tolerance was determined using MRS broth supplemented with 4% and 6.5% NaCl. In all tests, the appearance of turbidity after incubation was considered indicative of positive growth.

2.5. Technological Characterization of LAB Isolates

2.5.1. Acidifying and Proteolytic Activities

The technological properties of LAB isolates were evaluated through assessments of acidifying and proteolytic activities. Acidifying activity was determined using a modified method reported by Fawzi et al. [28]. Isolates were subcultured in MRS broth and incubated at 37 °C for 24 h; subsequently 100 μ L of each culture was inoculated into 10 mL of reconstituted skimmed milk (10% w/v) previously sterilized at 115 °C for 10 min. The inoculated tubes were incubated at 37 °C, and pH and titratable acidity (TA) were measured at 0, 2, 4, 6, and 24 h. Proteolytic activity was assessed by the agar disk diffusion method according to Raveschot et al. [29], using MRS and Plate Count Agar (PCA, Pasteur Institute of Algeria, Algiers, Algeria) media supplemented with 10% (w/v) skimmed milk. A 10 μ L aliquot of each overnight culture was placed on sterile Whatman paper disks (Fisherbrand™, Fisher Scientific, Waltham, MA, USA) positioned on the agar surface, and the plates were incubated at 37 °C for 24–48 h. Clear zones around the disks indicated proteolysis, and their diameters were measured to quantify enzymatic activity.

2.5.2. Antibacterial Potential

LAB isolates were screened for their antibacterial potential against twelve indicator strains, including six food-origin isolates and six reference (ATCC) strains. The food-origin isolates included *Escherichia coli* S55 and S216, *Enterobacter cloacae* S41 and S54, *Klebsiella pneumoniae* S20, and *Salmonella* spp. S16. The ATCC reference strains were *E. coli* ATCC 25922, *Klebsiella aerogenes* ATCC 10006, *Staphylococcus aureus* ATCC 25923, *Pseudomonas aeruginosa* ATCC 27853, *Enterococcus faecalis* ATCC 29212, and *Enterococcus faecalis* ATCC 29212. The antibacterial potential of LAB isolates was determined using the agar well diffusion assay following the procedure reported by Alghamdi et al. [30], with slight modifications. First, the indicator strains were cultured on nutrient agar at 37 °C for 24 h. After incubation, the overnight cultures were spread onto the surface of Mueller–Hinton agar (MHA, Pasteur Institute of Algeria, Algiers, Algeria) plates using sterile cotton swabs. Wells were then punched into the agar and filled with 100 μ L of the LAB cell suspension. The plates were incubated aerobically at 37 °C for 24 h. Clear inhibition zones around the

wells were measured and recorded as positive. The experiment was performed in triplicate, and the mean values were calculated.

2.6. Safety Assessment of Enterocin-Positive LAB Isolates

2.6.1. Detection of Antimicrobial Resistance Genes

LAB strains were subjected to PCR for the detection of genes conferring resistance to macrolide-lincosamide-streptogramin (*ermA*, *ermB*, *ermC*, *mefA/E*, and *msrA*) [31–34], tetracycline (*tetM*, *tetK*, *tetL*, and *tetO*) [35], vancomycin (*vanA* and *vanB*) [36], and oxazolidinone (*optrA* and *poxA*) [37,38], as described previously by Guo et al. [39]. Primers used and amplification conditions are presented in Table 1.

Tabela 1. Primers sequences and PCR conditions used for the detection of antimicrobial resistance, amino acid decarboxylase, and integron genes.

Genes	Primers (Sequence 5'–3')	Amplification Conditions	Size (bp)	References
<i>ermB</i>	F-GAAAAGGTACTCAACCAATA R-GTAACGGTACTTAAATTGTTTAC	94 °C/5 min; 35 cycles: 94 °C/30 s, 50 °C/30 s, 72 °C/1 min; 72 °C/5 min	142	[31]
<i>ermA</i>	F-TATCTTATCGTTGAGAAGGGATT R-CTACACTTGGCTTAGGATGAAA	95 °C/5 min; 30 cycles: 95 °C/30 s, 55 °C/30 s, 72 °C/30 s; 72 °C/5 min	139	[32]
<i>ermC</i>	F-CTTGTTGATCAGGATAATTTCC R-ATC TTTAGCAAACCCGTATTC	95 °C/5 min; 30 cycles: 95 °C/30 s, 55 °C/30 s, 72 °C/30 s; 72 °C/5 min	190	
<i>mefA/E</i>	F-AGTATCATTAATCACTAGTGC R-TTCTTCTGGTACTAAAAGTGG	93 °C/5 min; 35 cycles: 93 °C/1 min, 52 °C/1 min, 72 °C/1 min; 72 °C/5 min	348	[33]
<i>msrA</i>	F-GCAAATGGTGTAGGTAAGACAACT R-ATCATGTGATGTAAACAAAT	95 °C/3 min; 35 cycles: 94 °C/30 s, 55 °C/2 min, 72 °C/90 s; 72 °C/5 min	400	[34]
<i>tetL</i>	F-TCGTTAGCGTGCTGTCATTC R-GTATCCCAACCAATGTAGCCG		267	
<i>tetO</i>	F-AACTTAGGCATTCTGGCTCAC R-TCCCACTGTTCCATATCGTCA	94 °C/5 min; 30 cycles: 94 °C/30 s, 55 °C/30 s, 72 °C/1 min; 72 °C/5 min	515	[35]
<i>tetK</i>	F-TCGATAGGAACAGCAGTA R-CAGCAGATCCTACTCCTT		169	
<i>tetM</i>	F-GTGGACAAAGGTACAACGAG R-CGGTAAAGTTCGTCACACAC		500	
<i>vanA</i>	F-GGGAAAACGACAATTGC R-GTACAATGCGGCCGTTA	96 °C/2 min; 35 cycles: 94 °C/30 s, 50 °C/30 s, 72 °C/1 min; 72 °C/10 min	732	[36]
<i>vanB</i>	F-ATGGGAAGCCGATAGTC R-GATTTCGTTTCCTCGACC	94 °C/2 min; 30 cycles: 94 °C/1 min, 54 °C/1 min, 72 °C/1 min; 72 °C/10 min	635	
<i>optrA</i>	F-TACTTGATGAACCTACTAACCA R-CCTTGAACCTACTGATTCTCGG	94 °C/5 min, 35 cycles: 94 °C/30 s, 55 °C/30 s, 72 °C/1 min; 72 °C/5 min	422	[37]
<i>poxA</i>	F-AAAGCTACCCATAAAATATC R-TCATCAAGCTGTTTCGAGTTC	96 °C/2 min; 30 cycles: 96 °C/30 s, 50 °C/30 s, 72 °C/30 s; 72 °C/5 min	533	[38]
<i>hyl</i>	F-ACAGAAGAGCTGCAGGAAATG R-GACTGACGTCCAAGTTTCCAA	94 °C/5 min; 30 cycles: 94 °C/1 min, 54 °C/1 min, 72 °C/1 min; 72 °C/10 min	276	[40]
<i>esp</i>	F-AGATTTTCATCTTTGATTCTTGG R-AATTGATTCTTTAGCATCTGG		510	

Tabela 1. Cont.

Genes	Primers (Sequence 5'–3')	Amplification Conditions	Size (bp)	References
<i>hdc</i>	F-GGNATNGTNWSNTAYGAYMGNGCNGA R-ATNGCDATNGCNSWCCANACNCCRTA		372	
<i>ldc</i>	F-CAYRTNCCNGGNCAYAA R-GGDATNCCNGGNGGRTA	95 °C/10 min; 30 cycles: 95 °C/30 s, 53 °C/30 s, 72 °C 2 min. 72 °C 20 min.	1185	[41]
<i>odc</i>	F-TWYMAYGCNGAYAAACNTAYTTYGT R-ACRCANAGNACNCCNGGNGGRTANGG		1440	
<i>tdc</i>	F-TGGYTNGTNCCNCARACNAARCAITA R-ACRTARTCNACCATRTTAAARTCNCGG		825	
<i>int1</i>	F-GGGTCAAGGATCTGGATTTCG R-ACATGGGTGTAAATCATCGTC	95 °C 5 min; 30 cycles (94 °C 30 s, 62 °C 30 s, 72 °C 1 min); 72 °C 5 min	483	[42]
<i>int2</i>	F-CACGGATATGCGACAAAAAGGT R-GTAGCAAACGAGTGACGAAATG		788	

2.6.2. Detection of Virulence Genes

The molecular detection of virulence genes in LAB strains was performed using PCR, following the procedure reported by Vankerckhoven et al. [40]. Two virulence genes were targeted: *hyl* (hyaluronidase) and *esp* (enterococcal surface protein). The specific primer sequences and amplification conditions are provided in Table 1.

2.6.3. Detection of Genes Encoding Biogenic Amine-Producing Enzymes

Detection of biogenic amine biosynthesis genes in LAB isolates was performed using PCR according to the procedure described by Geniş et al. [41]. The genes investigated were *tdc* (tyrosine decarboxylase), *odc* (ornithine decarboxylase), *hdc* (histidine decarboxylase), and *ldc* (lysine decarboxylase). Primer sequences and PCR cycling conditions are listed in Table 1.

2.6.4. Detection of Integron Genes

The presence of *intI1* and *intI2* genes in LAB strains was determined by PCR using specific primers (Table 1), following the protocol reported by Mazel et al. [42].

2.7. Statistical Analysis

In vitro antibacterial activity assays were performed in triplicate and the obtained inhibition zone diameters were expressed as mean $\pm$ standard deviation. Statistical analysis was conducted using MedCalc® Statistical Software (version 23.1.6, MedCalc Software Ltd., Ostend, Belgium). Statistical significance of differences in the antibacterial potential among enterocin-positive LAB isolates was evaluated using the Kruskal–Wallis test, while variations in susceptibility among the tested indicator strains were analyzed using the Friedman test. Statistical significance was considered at $p < 0.05$, and values of $p < 0.01$ were regarded as highly significant.

3. Results and Discussion

3.1. Species Identification

3.1.1. Phenotypic Identification

In the present study, four Gram-positive, catalase-negative, non-motile bacterial strains were isolated from two artisanal dairy product samples and identified as presumptive LAB. The isolation of multiple LAB strains from each artisanal dairy product reflects diverse microbial communities typically associated with traditional dairy products. This richness is often attributed to the absence of standardized starter cultures and the influence of local

environmental and processing conditions [43]. The isolation of LAB from artisanal dairy products is consistent with previous studies highlighting the richness of traditional dairy matrices in LAB [6,8].

3.1.2. MALDI-TOF Identification

According to MALDI-TOF identification results, two isolates were identified as *Enterococcus faecalis*, one as *L. paracasei* subsp. *paracasei*, and one as *Lev. brevis*. The predominance of *Enterococcus* spp. is consistent with previous studies in dairy products [44,45]. The dominance of *E. faecalis* suggests that hygiene conditions or processing parameters may favor its persistence. This may be linked to its ability to form biofilms, contributing to its persistence in dairy processing environments [46]. The presence of *L. paracasei* and *L. brevis* in these samples underscores the diversity of the microbiota associated with artisanal dairy products. Unlike industrial fermentations that utilize defined starter cultures, artisanal processes rely on indigenous microbiota, which can result in microbial diversity and present opportunities for isolating strains with novel functional or technological properties. In fact, *L. paracasei* and *L. brevis* isolates are widely recognized for their probiotic potential and functional properties [5,7,18,47]. Their presence in both fresh cream and butter suggests that they may play a significant role in the fermentation ecology of these traditional dairy products.

Since *Enterococcus* species have been associated with antibiotic resistance and virulence traits in dairy settings [44], raising potential food safety concerns, only the non-enterococcal LAB isolates were selected for further phenotypic and genotypic characterization.

3.1.3. Detection of *Enterococcus*-Specific *tuf* Gene

To further confirm that the two selected isolates (*L. paracasei* S2 and *Lev. brevis* S62) did not belong to the genus *Enterococcus*, a PCR assay targeting the *tuf* gene specific to *Enterococcus* spp. was performed. The results showed no amplification of the *tuf* gene in either isolate. These findings confirm that the two isolates are non-enterococcal LAB, consistent with the MALDI-TOF identification results. Their identification as non-enterococcal LAB by both *tuf* gene-based PCR assay and MALDI-TOF MS demonstrates a strong concordance between molecular and proteomic methods. As reported by Ke et al. [26], the *tuf* gene-based PCR assay exhibits remarkable accuracy and specificity in distinguishing *Enterococcus* species from other closely related Gram-positive bacteria.

3.2. Detection of Enterocin-Encoding Genes

The production of bacteriocins by LAB is one of the most important criteria for probiotic strain selection [48]. Screening for enterocin-encoding genes revealed that both isolates carried enterocin genes. Each isolate harbored a single enterocin gene: the *entQ* gene was detected in *L. paracasei* S2, and the *entAS-48* gene in *Lev. brevis* S62 (Table 2). The other investigated enterocin genes, including *ent31* (*bac31*), *entA*, *entB*, *entL50A/B*, *entP*, *ent1071A/B*, *entX*, *entSE-K4*, *hirJ*M79, and *entL*, were not detected in the tested strains. *EntQ* is a class IIc, non-pediocin-like, linear one-peptide bacteriocin described in *E. faecium*, whereas *entAS-48* is a circular class IIc bacteriocin [13]. Their natural occurrence in LAB isolates with GRAS status is noteworthy, as both bacteriocins exhibit strong antimicrobial activity and remarkable stability under harsh conditions, including extreme pH, high temperature, and chemical denaturants [14]. In our previous study, these genes were identified for the first time in *L. paracasei* and *Lev. brevis* from artisanal dairy products made from raw cow milk without starter cultures, suggesting potential horizontal gene transfer mediated by mobile genetic elements from *Enterococcus* spp. [12]. Indeed, *entQ* is located on the 7.4 kb plasmid pCIZ2, and *entAS-48* is carried on the pMB2 plasmid [49,50], with demonstrated horizontal transferability across genera [49,51]. Furthermore, it has been reported that the

entAS-48 gene cluster has been successfully cloned into LAB strains, and that *entQ* can be heterologously expressed in *Lactobacillus sakei*.

Tabela 2. Phenotypic and MALDI-TOF identification of lactic acid bacteria isolated from Algerian artisanal dairy products.

Isolate Code	Sample	Cell Shape	Gram	Catalase	Oxydase	Motility	MALDI- TOF Identification	Enterocin Genes
S2	Fresh cream	Rod	+	—	—	—	<i>Lactocaseibacillus paracasei</i> subsp. <i>paracasei</i>	<i>entQ</i>
S44b	Fresh cream	Cocci	+	—	—	—	<i>Enterococcus faecalis</i>	ND
S26b	Butter	Cocci	+	—	—	—	<i>Enterococcus faecalis</i>	ND
S62	Butter	Rod	+	—	—	—	<i>Levilactobacillus brevis</i>	<i>entAS48</i>

(+): positive reaction; (—): negative reaction; ND: not determined.

3.3. Biochemical Characterization

LAB growth was evaluated under different temperatures, pH values, and NaCl concentrations (Table 3). Both strains grew at 37 °C, whereas *L. paracasei* S2 also grew at 45 °C. Regarding pH tolerance, *L. paracasei* S2 did not grow at pH 2 but grew at pH 3–5, while *Lev. brevis* S62 tolerated a wider acidic range (pH 2–5). It has been shown that LAB exhibit significantly reduced viability under highly acidic conditions, especially at pH values below 2.5 [4]. It has also been reported that the capacity of LAB to persist in such environments is strain-dependent and involves multiple adaptive mechanisms, among which the F₀F₁-ATPase complex plays a pivotal role in facilitating acid stress resistance and promoting the survival of LAB isolates [52]. In terms of salt tolerance, the *Lev. brevis* S62 isolate demonstrated the ability to grow at a NaCl concentration of 4%, whereas none of the isolates exhibited growth at the higher concentration of 6.5%. Although research specifically focused on the growth of *Lev. brevis* at 6.5% NaCl is limited, Bentahar et al. [7] reported that two *Lev. brevis* isolates were able to grow across a range of NaCl concentrations (2% to 10%), with a progressive decline in growth observed from 3% onward.

Tabela 3. Biochemical and technological properties of enterocin-positive lactic acid bacteria.

Strains	Growth at T (°C)			Growth at pH				Growth at NaCl (%)		Proteolytic Power	
	15 °C	37 °C	45 °C	2	3	4	5	4%	6.5%	MRS Medium	PCA Medium
<i>L. paracasei</i> S2	—	+	+	—	+	+	+	—	—	22	22
<i>Lev. brevis</i> S62	—	+	—	+	+	+	+	+	—	18.5	Growth

(+): positive reaction; (—): negative reaction; T (°C): temperature in °C.

3.4. Biotechnological Properties

3.4.1. Acidifying and Proteolytic Activities

The results of acidification assays showed that *L. paracasei* S2 reduced the pH of the medium from 6.7 to 5.45 over 24 h, accompanied by an increase in TA from 14 °D to 38.5 °D (Figure 1). *Lev. brevis* S62 exhibited a comparable acidifying capacity, lowering the pH from 6.68 to 5.40 and increasing TA from 13 °D to 32 °D (Figure 2). Both strains displayed proteolytic activity on MRS medium, with *L. paracasei* S2 showing slightly higher activity. On PCA medium, *L. paracasei* S2 demonstrated clear proteolytic activity, whereas *Lev. brevis* S62 showed limited growth and minimal proteolysis. These results confirmed that both strains possess functional acidification and proteolytic systems characteristic of LAB. Previous studies have shown that acidifying and proteolytic capacities vary markedly between LAB species and even among strains of the same species [5,8]. The moderate but efficient acidification observed for both isolates agrees with earlier reports describing *Lactobacillus* strains as exhibiting moderate to strong acidifying power [5,53], while the

strain-dependent variability observed between *Lev. brevis* isolates has also been widely reported [54]. Likewise, the higher proteolytic performance of *L. paracasei* S2 compared with *Lev. brevis* S62 is consistent with evidence that *L. paracasei* often exhibits strong casein-hydrolyzing ability [47], whereas proteolysis within *Lev. brevis* is more heterogeneous across strains [55]. Together, the acidification and proteolytic profiles of the two isolates reflect the typical metabolic diversity observed within LAB and support their potential technological relevance in dairy fermentation.

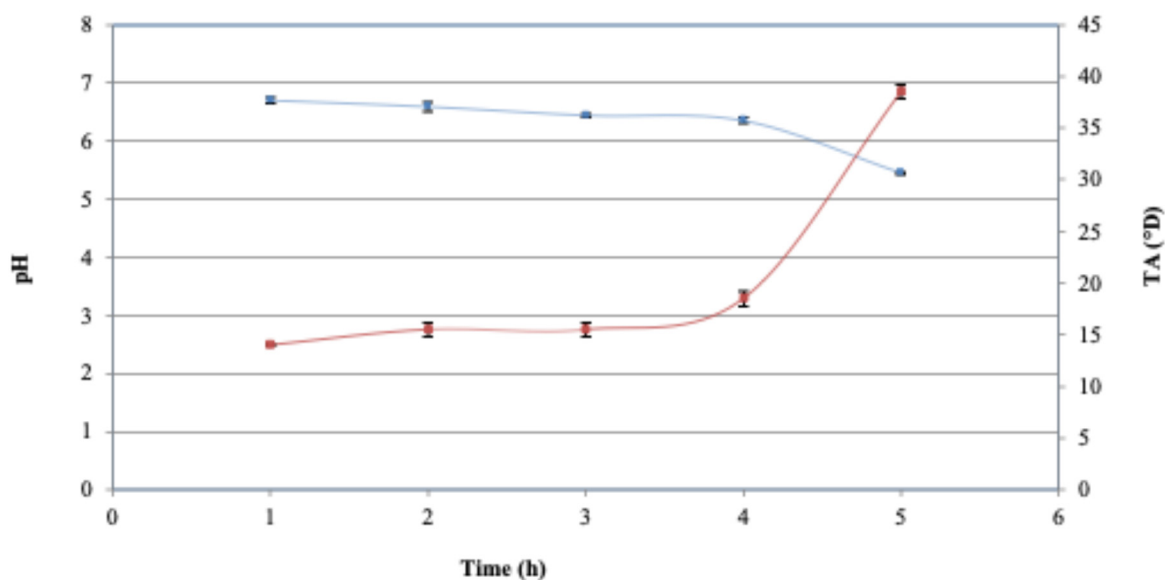


Figure 1. Changes over time of pH (blue line) and Titratable acidity (TA (°D), red line) of *L. paracasei* S2 over time. Error bars represent standard deviation from triplicate measurements.

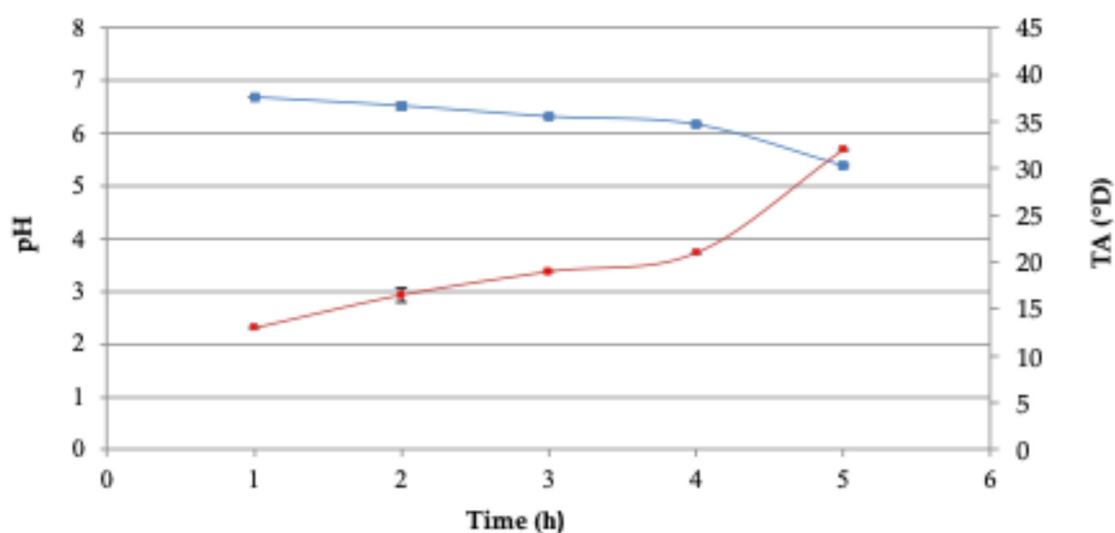


Figure 2. Changes over time of pH (blue line) and Titratable acidity (TA (°D), red line) of *Lev. brevis* S62 over time. Error bars represent standard deviation from triplicate measurements.

3.4.2. Antibacterial Potential

The results of the antimicrobial activity of both LAB strains against the tested indicator strains are reported in Table 4. Both enterocin-positive LAB strains demonstrated activity against most tested microorganisms, with inhibition zones ranging from <6 to 27.67 ± 2.31 mm. *L. paracasei* S2 generally showed stronger inhibition, particularly against Gram-positive ATCC strains such as *S. aureus* ATCC 25923, *E. faecalis* ATCC 29212, and *E.*

faecium ATCC 6057, whereas *Lev. brevis* S62 exhibited moderate activity, notably against *E. coli* ATCC 25922. Both strains produced larger and more consistent inhibition zones against ATCC reference strains compared with food-origin isolates, which often displayed smaller or no inhibition (<6 mm). Differences in inhibition between the two LAB strains were statistically significant for most strains (*p* values ranging from 0.03 to 0.05). These findings are consistent with previous studies reporting strong antibacterial activity of *L. paracasei* and *Lev. brevis* against Gram-positive pathogens [3,56]. The pronounced activity against Gram-positive ATCC strains might be attributed to the action of enterocins entAS-48 and entQ, which disrupt cytoplasmic membrane integrity and form lethal pores [57,58]. In contrast, Gram-negative food-origin isolates such as *E. coli*, *Salmonella*, and *Klebsiella* were less sensitive, probably due to the protective outer membrane.

Tabela 4. Antibacterial potential of enterocin-positive lactic acid bacteria.

Isolates	Antimicrobial Potential (mm)												<i>p</i> Value
	<i>E. coli</i> S216	<i>E. coli</i> S55	<i>E. cloacae</i> S41	<i>E. cloacae</i> S54	<i>K. pneumoniae</i> S20	<i>Salmonella</i> spp. S16	<i>E. coli</i> ATCC 25922	<i>K. aerogenes</i> ATCC 10006	<i>S. aureus</i> ATCC 25923	<i>Paerugenosa</i> ATCC 27853	<i>E. faecalis</i> ATCC 29212	<i>E. faecium</i> ATCC 6057	
<i>L. paracasei</i> S2	13.67 ± 0.58 a/c	<6 a/e	9.67 ± 0.58 b/d	<6 a/e	13.00 ± 0.00 b/c	13.00 ± 0.00 b/c	21.33 ± 1.53 a/b	21.00 ± 0.00 b/b	23.00 ± 0.00 b/b	27.67 ± 2.08 b/a	27.00 ± 1.00 b/a	22.67 ± 2.31 b/b	<0.00001
<i>Lev. brevis</i> S62	15.00 ± 0.00 b/cd	11.67 ± 0.58 b/e	<6 a/g	9.00 ± 0.00 b/f	12.00 ± 0.00 a/e	12.00 ± 0.00 a/e	24.33 ± 0.58 a/b	16.33 ± 0.58 a/c	18.67 ± 0.58 a/b	18.67 ± 2.52 a/b	14.00 ± 0.00 a/d	14.00 ± 1.00 a/d	<0.00001
<i>p</i> value	0.03	0.034	0.03	0.03	0.03	0.03	0.05	0.03	0.03	0.05	0.04	0.05	

Results are expressed as mean ± standard deviation (mm). Different letters indicate significant differences at *p* < 0.05 among isolates.

3.5. Safety Assessment of Enterocin-Positive LAB

3.5.1. Detection of Antibiotic Resistance Genes

Both LAB isolates were screened for the presence of common antibiotic resistance genes to evaluate their safety for potential use in food biopreservation. None of the tested antibiotic resistance genes were detected in the isolates, indicating a safe antimicrobial profile. This finding aligns with EFSA guidelines, which recommend that lactobacilli intended for human consumption must be free of acquired or transferable resistance determinants [59]. Several studies have highlighted the importance of monitoring antibiotic resistance genes in LAB from raw milk and artisanal dairy products, as these bacteria can act as reservoirs for resistance genes and potentially transfer them to other commensal or pathogenic bacteria in the human gut [15,19,20]. Notably, a study by EFSA reported the detection of resistance to clindamycin and ampicillin in *Lev. brevis* NCIMB 42129, illustrating that some strains may carry acquired resistance even within generally safe species [59]. Most antibiotic resistance genes, such as those conferring resistance to clindamycin and ampicillin, are associated with horizontally acquired elements and can potentially be transferred between bacteria [59]. Indeed, the horizontal transfer of tetracycline resistance genes, such as *tetK*, *tetS*, and *tetM*, has been demonstrated from certain *Lactobacillus* strains to pathogens like *L. monocytogenes*, underscoring the potential for dissemination of these resistance determinants [20].

3.5.2. Detection of Virulence Genes

In the present study, screening for the virulence genes *hyl* and *esp* confirmed their absence in the two tested strains. The safety of LAB from artisanal dairy products has been extensively evaluated through the assessment of virulence factors [17,21]. It has been reported that certain strains, including *L. paracasei* AD22, *Lev. brevis* DPL5, and *L. paracasei* SLP16, do not possess virulence genes, making them appropriate for food-related applications [60–62]. Similarly, *L. paracasei* IDCC 3401 has been demonstrated to not carry virulence determinants [63]. The absence of *hyl* and *esp* in the strains evaluated in this study reinforces their biosafety profile and supports their potential use in the

food industry. In contrast, strains that harbor both virulence and bacteriocin genes are considered potentially unsafe, as they may pose health risks and are therefore unsuitable for technological applications.

3.5.3. Detection of Biogenic Amines Genes

The presence of biogenic amines in dairy products is a major food safety concern due to their involvement in foodborne intoxications and adverse physiological effects, such as headaches, hypertension, and allergic reactions [64]. Accordingly, the detection of biogenic amine-producing strains is a critical criterion when selecting LAB for starter or biopreservative cultures, with the aim of producing high-quality foods with reduced biogenic amine content [65,66]. In this study, the LAB isolates were screened by PCR for four key decarboxylase genes responsible for tyramine, putrescine, histamine, and cadaverine production. None of the tested genes were detected in either strain, supporting their suitability for application in food systems. This is particularly relevant for artisanal fermented products, where uncontrolled processing can favor the accumulation of toxic biogenic amines. The ability to produce biogenic amines is generally strain-specific, and while several studies have detected decarboxylase genes such as *tdc* and *hdc* in *Lactobacillus* spp. from fermented foods [16,18,22], the absence of these genes in the present strains highlights their favorable safety profile.

3.5.4. Detection of Integron Genes

In the present study, *L. paracasei* S2 and *Lev. brevis* S62 were found to lack the integron genes *intI1* and *intI2*. This absence may be related to differences in genome organization and the distribution of mobile genetic elements, which are commonly involved in the horizontal acquisition and dissemination of various functional genes, including those conferring antibiotic resistance, metabolic traits, or stress adaptation [67,68]. The genomic architecture of LAB appears less favorable for the integration of integrons typically observed in Gram-negative bacteria. While *intI1* and *intI2* genes have been reported in both Gram-negative and Gram-positive bacteria [69,70], no study has reported the presence of these integron genes in LAB isolated from artisanal dairy products [71].

4. Conclusions

This study demonstrates that *L. paracasei* S2 and *Lev. brevis* S62, isolated from artisanal dairy products, exhibit notable technological and antibacterial potential. Both strains displayed effective acidification, proteolytic activity, and broad-spectrum antimicrobial activity, associated with the presence of the enterocin genes *entQ* and *entAS-48*. A comprehensive safety assessment revealed the absence of virulence determinants, antibiotic resistance genes, biogenic amine-producing genes, and integrons, indicating a minimal risk of horizontal transfer of undesirable traits. These findings highlight the favorable biosafety and functional profiles of the two isolates, supporting their potential application as starter or biopreservative cultures in artisanal dairy fermentations to improve product quality while mitigating food safety risks.

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Chapter 3

***Co-occurrence of enterocin genes with hyl and
msrA genes in Lacticaseibacillus paracasei isolates
with antibacterial potential against multidrug
resistant foodborne pathogens***

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Co-occurrence of enterocin genes with hyl and msrA genes in *Lacticaseibacillus paracasei* isolates with antibacterial potential against multidrug resistant foodborne pathogens

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**Co-occurrence of enterocin genes with *hyl* and *msrA* genes in *Lactocaseibacillus*
paracasei isolates with antibacterial potential against multidrug resistant foodborne
pathogens**

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Abstract

The increasing prevalence of multidrug resistance (MDR) in foodborne pathogenic bacteria indicates the urgent need for alternative antimicrobial agents. This study aimed to evaluate the antibacterial potential of indigenous lactic acid bacteria (LAB) from artisanal dairy products and to investigate the presence of enterocin genes in strains demonstrating strong antibacterial activity. Their safety profile was also assessed. A total of seven LAB were isolated from three traditional dairy product samples and identified using phenotypic and proteomic methods. The strains were identified as *L. paracasei* (n=5) and *Lev. brevis* (n=2) using MALDI-TOF MS. The isolates exhibited species- and strain-dependent antibacterial activity, with several strains showing strong antibacterial activity against all the tested MDR pathogens. PCR analysis revealed the presence of *entAS48* in *L. paracasei* S8 and *ent1071A/B* in *L. paracasei* S93, suggesting the dissemination of these enterocin genes beyond enterococcal hosts. Molecular safety assessment showed a favorable safety profile of enterocin-negative isolates, highlighting their potential application in the food industry as natural bio-preservatives, although *msrA* and *hyl* was detected in the enterocin-positive isolates. This is the first study reporting the detection of *ent1071A/B* gene in non-enterococcal LAB. These findings indicate that Algerian artisanal dairy products harbor LAB with promising antibacterial potential for bioprotective applications, while further genomic and functional investigations are needed to further elucidate the genetic context, transferability, and in situ expression of these determinants.

Keywords: antibacterial potential, enterocin genes, *Lactocaseibacillus paracasei*, *Levilactobacillus brevis*, foodborne pathogens.

1. Introduction

Food safety is a global public health and economic concern, leading to 600 million illnesses, 420,000 deaths annually, and \$110 billion in costs for low- and middle-income countries (WHO, <https://www.who.int/news-room/fact-sheets/detail/food-safety>). Foodborne pathogens are the primary agents responsible for these illnesses, often transmitted through contaminated food such as eggs, meat, and milk (Kurćubić et al., 2025; Lima et al., 2023; Sievers et al., 2025). A major challenge to food safety today is the increasing prevalence of antimicrobial resistance (AMR), which undermines treatment efficacy and leads to more severe and difficult-to-treat foodborne infections (Lima et al., 2023). Of particular concern is the emergence and spread of multidrug-resistant (MDR) *Enterobacteriaceae*, a family of Gram-negative bacteria that includes common foodborne pathogens such as *Salmonella typhimurium* and *Escherichia coli* (Beyene et al., 2024; Garbaj et al., 2022). These organisms are increasingly resistant to multiple classes of antibiotics, including those considered last-resort treatments, such as third-generation cephalosporins and fluoroquinolones (Kurćubić et al., 2025). The presence of MDR *Enterobacteriaceae* in the food chain poses a serious risk to both human and animal health (Samtiya et al., 2022), and underscores the urgent need for developing effective control strategies across the One Health spectrum.

In response to this crisis, there is growing interest in identifying alternative natural antimicrobial agents that are both effective and safe for use in food systems. Among these, lactic acid bacteria (LAB) have gained significant attention due to their long history of safe use in fermented foods, their generally recognized as safe (GRAS) status, and their ability to inhibit pathogenic microorganisms, including MDR bacteria (Hamed et al., 2025; Ghane et al., 2021). LAB can suppress pathogens by producing organic acids, hydrogen peroxide, and bacteriocins, ribosomally synthesized antimicrobial peptides that target closely related or even unrelated bacterial species (Chen et al., 2025; Rishi et al., 2017). Of particular interest are

enterocins, a class of bacteriocins originally described in *Enterococcus* species, which have demonstrated potent inhibitory activity against a broad range of foodborne pathogens, including *Listeria monocytogenes* (Wu et al., 2022). However, safety concerns such as the presence of virulence factors, biogenic amine production, and antibiotic resistance have limited the industrial use of enterocin-producing *Enterococcus* spp. isolates (Hanchi et al., 2018). The recent occurrence of enterocin genes in non-enterococcal LAB, such as *Lacticaseibacillus paracasei* (*L. paracasei*) and *Levilactobacillus brevis* (*Lev. brevis*) known for their GRAS status (Belkacem et al., 2025; Belkacem et al., 2026), highlights the potential of horizontal gene transfer (HGT) events in artisanal dairy microbiomes and expands the pool of GRAS enterocin-producing strains suitable for biopreservation applications.

Artisanal dairy products, especially those derived from raw milk, represent a rich and underexplored source of microbial biodiversity and cultural heritage (Psomas et al., 2023; Silva et al., 2023). Unlike industrial dairy fermentations that rely on standardized starter cultures, artisanal products often undergo spontaneous fermentation, resulting in complex and dynamic microbial ecosystems dominated by LAB (Bendimerad et al., 2012; Mangia et al., 2014). They often harbor LAB with unique technological characteristics adapted to local production environments (Bentahar et al., 2024; Bouchibane et al., 2023; Hadeif et al., 2023). These bacteria contribute not only to the development of desirable organoleptic properties but also enhance the safety and shelf-life of fermented foods (Chen et al., 2025).

Safety assessment of LAB is essential before their application in food systems (EFSA panel on FEEDAP et al., 2021), especially for those derived from raw milk and artisanal dairy products. Although *Lactobacillus* spp. isolates are generally considered as GRAS, some strains may harbor antimicrobial resistance genes (ARGs), virulence determinants, or biogenic amine (BA)-producing genes (Ashwini et al., 2023; Barbieri et al., 2019; EFSA,

2024; Colautti et al., 2022). ARGs can potentially be horizontally transferred to commensal or pathogenic bacteria in the human gut, raising public health concerns (Yang & Yu, 2019). Similarly, genes encoding virulence factors and BA-producing enzymes, including tyrosine, histidine, lysine, and ornithine decarboxylases, may compromise the safety of fermented products (Ladero et al., 2010a; Colautti et al., 2022). Therefore, comprehensive molecular screening for ARGs, virulence genes, and BA-producing genes is essential to assess the biosafety of LAB isolates. In this context, the present study aimed to (i) isolate and identify LAB strains from Algerian artisanal dairy products, (ii) assess their antibacterial activity against MDR foodborne pathogens, (iii) investigate the presence of enterocin genes in non-enterococcal LAB isolates exhibiting significant antibacterial potential, and (iv) perform a comprehensive molecular safety assessment by screening for ARGs, virulence determinants, and BA-producing and integron genes.

2. Material and methods

2.1. Isolation of LAB

In this study, three artisanal dairy product samples were used as the source for LAB isolation. These products include fresh cream, Lben, and butter. These products were made from raw cow milk without the addition of starter cultures, according to the previously described method (Belkacem et al., 2026). These samples were aseptically collected from two different dairy farms in Mostaganem province, Algeria, transported under refrigeration, and processed within 24 h after collection. LAB were isolated following the method described by Ghane et al. (2021). Briefly, 1 g of each sample was aseptically suspended in 90 mL of TSE buffer. After homogenization, serial tenfold dilutions (10^{-1} to 10^{-7}) were prepared, and 0.1 mL of each dilution was spread onto de Man, Rogosa, and Sharpe (MRS) agar. The inoculated plates

were then incubated at 37 °C under anaerobic conditions. After incubation, colonies with distinct morphologies were selected and purified through repeated streaking on MRS agar.

2.2. LAB identification

2.2.1. Preliminary identification of LAB

The preliminary identification of LAB isolates was carried out according to the method described by Atfaoui et al. (2022). Colonies that were Gram-positive, non-spore-forming, and negative for both oxidase and catalase tests were considered presumptive LAB. The selected isolates were preserved in MRS broth supplemented with 30% (v/v) glycerol and stored at -20 °C for future analyses.

2.2.2. Identification of LAB by MALDI-TOF MS

Prior to MALDI-TOF MS analysis, LAB isolates were grown on Columbia agar supplemented with 10% sheep blood at 37°C for 24 to 48h. Identification of LAB isolates was performed by using MALDI Biotyper® Sirius System (Bruker, Ettlingen, Germany). Briefly, a small amount of fresh bacterial biomass was transferred onto a stainless-steel target plate and evenly spread to form a thin film. One µL of α -cyano-4-hydroxycinnamic acid matrix solution was then added to each spot and allowed to air-dry at room temperature. The dried target plate was then introduced into the MALDI-TOF MS and the obtained spectral data were analyzed using the manufacturer's software and compared with the Bruker reference spectra in the database for genus- and species-level identification.

2.3. Antimicrobial potential of LAB

The antagonistic activity of LAB isolates was evaluated using the agar well diffusion assay against a panel of microorganisms following the method described by Asha et al. (2024) with slight modifications. The indicator strains were first cultured on nutrient agar and incubated at

37 °C for 24h. Overnight cultures were then uniformly spread onto fresh Mueller-Hinton (MH) agar plates using sterile cotton swabs. Wells were aseptically created and subsequently filled with 100 µL of LAB cell suspension. Plates were incubated aerobically at 37 °C for 24h, and inhibition zones larger than 6 mm were considered indicative of antagonistic activity. Antagonistic activity tests were performed in triplicate, and mean inhibition zone diameters were calculated. The indicator strains included six ATCC isolates and eight pathogenic strains. The ATCC isolates included *Klebsiella aerogenes* (*K. aerogenes*) ATCC 10006, *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, *Enterococcus faecalis* (*E. faecalis*) ATCC 29212, *E. faecium* ATCC 6057, *Staphylococcus aureus* (*S. aureus*) ATCC 25923, and *Escherichia coli* (*E. coli*) ATCC 25922. However, pathogenic strains included *E. coli* S55 and S216, *Enterobacter cloacae* (*E. cloacae*) S41 and S54, *K. pneumoniae* S20, *Salmonella typhimurium* (*S. typhimurium*) S16, *S. enteritidis* S27, and *Listeria monocytogenes* (*L. monocytogenes*) S61. The antimicrobial susceptibility testing of most of these pathogenic bacteria were previously studied against many antimicrobials including those of critical importance (Benameur et al., 2018a; Benameur et al., 2018b; Benameur et al., 2021) and their AMR profile is displayed in Table 1. The tested isolates displayed a MDR phenotype. Most of the antibiotics to which they were resistant are listed by the WHO as critically important for human and veterinary medicine. In addition, some isolates carried *bla*_{CTX-M1} and *qnr* genes, including *E. coli* S55 and S216 and *E. cloacae* S54.

2.4. Detection of enterocin genes

Genomic DNA was extracted from fresh LAB cultures using the boiling method as described by Singh et al. (2022). Briefly, a loopful of LAB cells was suspended in 100 µL of sterile distilled water, boiled at 100 °C for 10 min, cooled on ice, and centrifuged at 10000 × g for 5 min; the supernatant was used as template DNA. The presence of enterocin genes in LAB isolates was investigated by PCR as described by Lengliz et al. (2021). Twelve enterocin

genes were targeted in this study, including *hirJM79*, *entB*, *entA*, *entL50A/B*, *ent31* (*bac31*), *entP*, *entQ*, *entAS48*, *entX*, *entSE-K4*, *ent1071A/B*, and *entL*. The specific primers and PCR amplification conditions applied in this study were as previously described by Belkacem et al. (2025).

2.5. Safety characterization of LAB

2.5.1. Detection of antimicrobial resistance genes

LAB strains were analyzed by PCR for the detection of resistance determinants of tetracycline (*tetM*, *tetK*, *tetL*, and *tetO*), vancomycin (*vanA* and *vanB*), macrolide-lincosamide-streptogramin (*ermA*, *ermB*, *ermC*, *mefA/E*, and *msrA*), and oxazolidinone (*optrA* and *poxA*) following the previously described method (Guo et al. 2017). Primers and the amplification conditions used were as previously reported by Belkacem et al. (2026).

2.5.2. Screening for the virulence genes

Molecular characterization of virulence genes in LAB isolates was performed using PCR, as previously described by Vankerckhoven et al. (2004). The virulence genes evaluated in this study were *hyl* (hyaluronidase) and *esp* (enterococcal surface protein). All primers and amplification conditions employed were as described by Belkacem et al. (2026).

2.5.3. Detection of BA genes

LAB isolates were also tested for the presence of BA-producing genes using PCR, following the method described by Geniş, et al. (2024). The genes targeted included tyrosine decarboxylase (*tdc*), ornithine decarboxylase (*odc*), histidine decarboxylase (*hdc*), and lysine decarboxylase (*ldc*). Details of the primers and PCR amplification conditions were as described by Belkacem et al. (2026).

2.5.4. Detection of integron genes

All LAB strains were screened for the presence of *intI1* and *intI2* genes using PCR, following the method described by Mazel et al. (2000). The amplification conditions and primers used were as previously reported (Belkacem et al., 2026).

2.6. Statistical analysis

Antibacterial potential of LAB isolates was performed in triplicate and the obtained inhibition zone diameters are expressed as mean $\pm$ standard deviation. Statistical analysis of the obtained antibacterial activity results was performed using MedCalc® Statistical Software (version 23.1.6, MedCalc Software Ltd, Ostend, Belgium). Statistical significance of differences in the antibacterial potential between LAB isolates was determined using the Kruskal–Wallis test. The differences were considered significant at *P* value <0.05 and extremely significant at *P* value < 0.01 .

3. Results and discussion

3.1. LAB isolation and identification

3.1.1. Isolation and phenotypic identification

In the current study, seven LAB strains were isolated from three Algerian traditional dairy product samples: one from fresh cream, three from Lben, and three from butter. Preliminary screening confirmed characteristics typical of LAB: all isolates were Gram-positive, oxidase-negative, catalase-negative, non-motile, and non-spore-forming, as shown in Table 2. Morphological observations revealed variations in colony size, shape, and color, although all isolates were rod-shaped. In general, the colonies appeared white, larger, convex, and circular, exhibiting a smooth, creamy, and slightly slimy texture. Additionally, the isolates were capable of growing under anaerobic conditions. Based on these phenotypic characteristics, the LAB isolates were identified at the genus level as *Lactobacillus*. These findings align with

previous reports highlighting artisanal dairy products as a valuable source of LAB (Bendimerad et al., 2012; Ghalouni et al., 2018; Terzic-Vidojevic et al., 2014). Indeed, several studies have documented the isolation of LAB from Algerian artisanal dairy products, including Lben (Mangia et al., 2014; M'hamed et al., 2022), butter (Bentahar et al., 2024), and fresh cream (Belkacem et al. 2025).

3.1.2. MALDI-TOF identification

MALDI-TOF identification results showed that, out of the seven isolated LAB, five were identified as *L. paracasei* subsp. *paracasei* and two as *Lev. brevis*. The distribution of LAB species across the different dairy products is presented in Table 2. *L. paracasei* was the dominant species, being isolated from all the three types of artisanal dairy products examined. All isolates obtained from Lben were identified as *L. paracasei* and two out of the three isolates from butter were identified as *Lev. brevis*. The predominance of *L. paracasei* and *Lev. brevis* in Lben and butter samples, respectively, suggests a product-specific ecological niche. These findings are consistent with earlier reports describing *Lacticaseibacillus* and *Levilactobacillus* species as dominant members of the microbiota in traditional dairy products produced from raw cow milk (Belkacem et al. 2025; Bentahar et al., 2024).

3.2. Antimicrobial potential of LAB

The emergence and dissemination of MDR foodborne pathogens within the food chain highlight the urgent need for alternative antimicrobial strategies that are safe, natural, and sustainable. Seven LAB isolates were assessed in this study for their antibacterial potential against a panel of microorganisms, including MDR *Enterobacteriaceae* strains of food origin. The antimicrobial activity results are presented in Table 3. All LAB isolates exhibited inhibitory activity against the majority of the tested isolates, with inhibition zone diameters

ranging from 8.00 ± 0.00 to 32.00 ± 0.00 mm, indicating a pronounced but strain-dependent antagonistic effect ($P < 0.05$). Among the reference strains, *S. aureus* ATCC 25923 and *P. aeruginosa* ATCC 27853 were the most susceptible, particularly to *L. paracasei* S107 and S94 ($P < 0.01$), respectively, whereas *K. aerogenes* ATCC 10006 showed comparatively lower sensitivity. MDR Enterobacteriaceae isolates were also susceptible to the majority of the tested LAB isolates. However, notable strain-specific inhibition was observed, such as the selective antagonistic activity of *L. paracasei* S93 against *S. typhimurium* S16 ($P < 0.05$) and the consistent inhibition of *L. monocytogenes* S61 by most LAB isolates. Overall, *L. paracasei* strains displayed broader and stronger antimicrobial activity than *Lev. brevis* isolates ($P < 0.05$). These findings are in agreement with previous studies reporting that LAB-mediated antimicrobial activity is highly strain-specific and largely attributed to the production of organic acids, hydrogen peroxide, and bacteriocin-like substances (Asha et al., 2024; Rishi et al., 2017; Zhang et al., 2019). The observed higher susceptibility of Gram-positive bacteria compared to Gram-negative MDR Enterobacteriaceae aligns with previous studies and is generally attributed to structural differences in the bacterial cell envelope (Belkacem et al., 2026). Overall, the results highlight the potential of the isolated LAB strains as promising bioprotective agents for mitigating the spread of MDR foodborne pathogens.

3.3. Detection of enterocin-encoding genes

The screening of LAB isolates for bacteriocin-encoding genes is critical for evaluating their potential as natural bio-preservatives in food applications (Choi et al., 2021). LAB isolates that exhibited antibacterial activity were assessed by PCR to detect the presence of enterocin-encoding genes. PCR analysis revealed that, among the seven tested isolates, two carried enterocin genes: *L. paracasei* S8 harbored *entAS-48* and *L. paracasei* S93 carried *ent1071A/B* (Table 2). In contrast, *Lev. brevis* isolate did not carry any of the enterocin genes tested.

Furthermore, the remaining 10 enterocin genes (*entA*, *entB*, *entL*, *entL50A/B*, *entP*, *entQ*, *entSE-K4*, *entX*, *ent31*, *hirJM79*) were not detected in any of the tested LAB isolates. EntAS-48 and ent1071A/B belong to distinct classes: entAS-48 is a circular Class IIc bacteriocin with broad-spectrum activity, primarily acting through cytoplasmic membrane permeabilization and pore formation (Grande Burgos et al., 2014; Wu et al., 2022), whereas ent1071A/B is a two-peptide Class IIb bacteriocin that functions synergistically to disrupt target cell membranes (Nissen-Meyer et al., 2010). Enterocins are generally specific to *Enterococcus* genus, and *entAS-48* and *ent1071A/B* were originally identified in *E. faecalis* (Franz et al., 2002; Fernández et al., 2008). However, their occurrence in *L. paracasei* may reflect HGT events. In fact, the co-existence of *Enterococcus* and *Lactobacillus* isolates in artisanal dairy products and the presence of enterocin genes in enterococcal isolates from such products have been widely documented (Acero-Pimentel et al., 2024; Hadeef et al., 2023; Silva et al., 2023; Teso-Pérez et al., 2024; Merzoug et al., 2019). It has also been demonstrated that the *entAS-48* gene is carried on the pMB2 plasmid (Martínez-Bueno et al., 1994), and the *ent1071A/B* gene on the 50-kbp pEF1071 plasmid (Balla & Dicks, 2005). In addition, both genes have been shown to be transferable across LAB genera (Balla et al., 2000; Fernández et al., 2007; Franz et al., 2002). Our recent work also reported the detection of the *entAS-48* and *entQ* genes in *L. paracasei* and *Lev. brevis* strains isolated from artisanal dairy products (Belkacem et al., 2025; Belkacem et al., 2026). Notably, this study represents the first report of *ent1071A/B* gene in non-enterococcal LAB, highlighting the expanding diversity of enterocin genes beyond traditional enterococcal hosts.

The strong antibacterial activity observed in the strains *L. paracasei* S8 and S93 against the tested microorganisms may be attributed to the production of the *entAS-48* and *ent1071A/B* peptides. Indeed, it has been shown that both entAS-48 and ent1071A/B exhibit strong activity against Gram-positive pathogens, including *L. monocytogenes* and *S. aureus* (Balla et

al., 2000; Cheriet et al., 2023; Franz et al., 2002). However, the strong inhibitory activity of enterocin-negative isolates indicates that other mechanisms, such as organic acid production or uncharacterized bacteriocins, may also contribute to pathogen suppression. It has also been reported that these bacteriocins exhibit exceptional structural stability under heat, acidic and alkaline pH conditions, as well as chemical stress (Grande Burgos et al., 2014; Nissen-Meyer et al., 2010), indicating their strong potential as natural food preservatives. Accordingly, the natural occurrence of genes encoding such enterocins in LAB with GRAS status provides further support for their suitability in food preservation.

3.4. Safety assessment of LAB isolates

3.4.1. Detection of antimicrobial resistance genes

Several studies have reported that raw cow milk can contain antimicrobial resistant *Lactobacillus* spp. isolates, potentially acting as vectors for the transfer of ARGs to commensal bacteria in the human gut (Ashwini et al., 2023). Therefore, monitoring the presence of ARGs in lactobacilli from artisanal dairy products made from raw cow milk is essential for evaluating their safety profiles prior to their industrial application as food biopreservatives. The EFSA guidelines recommend that lactobacilli intended for human consumption be genetically screened to confirm the absence of transferable ARGs (EFSA BIOHAZ Panel et al., 2023). The results of this study showed the absence of the investigated ARGs in all the isolated LAB strains, except for the *ent1071A/B*-positive *L. paracasei* S93 which was found to carry *msrA* (Table 2). This ARG encodes a protein from the ABC transporter family that functions as an efflux pump, providing resistance by actively removing macrolides and streptogramin B from the bacterial cell (Portillo et al., 2000). While some related genes, such as *msrC* in *E. faecium*, are intrinsic chromosomal features, the resistance-associated *msrA* is typically found on large plasmids or mobile genetic elements in Gram-

positive bacteria (Portillo et al., 2000). The occurrence of *msrA* in enterocin-positive *L. paracasei* strain suggests a HGT event originating from an *Enterococcus* donor. This hypothesis is supported by the fact that enterococci are known reservoirs for acquired resistance and frequently harbor broad-host-range plasmids, such as *pAM β 1*, which are capable of replicating effectively within various LAB, including *Lactobacillus* species (Kazi et al., 2022). In fact, genomic analysis has revealed that the most closely related genetic context to the *msrA* operon of *E. faecalis* is found in *L. casei* ATCC 334 (Zhao et al., 2010). This latter has been found to share an identical cluster of five genes in its genomic region, which likely provides a compatible environment for the stable integration of enterococcal DNA (Zhao et al., 2010). The presence of ARGs in LAB can also be attributed by mutation-driven mechanisms that modify intrinsic resistance traits as previously suggested (Rozman et al., 2023).

Several studies have reported that LAB from fermented dairy products carry ARGs and may contribute to their horizontal transfer (Floris et al., 2025; Obioha et al., 2023; Yang & Yu, 2019). In their study, Ashwini et al. (2023), reported the detection of *msrA*, *msrA/B*, *msrC*, *ermB*, and *mefA/E* in several *Lactobacillus* sp. isolates. Similarly, another study reported the presence of *ermB*, *ermF*, *tetS*, *tetW*, *tetO*, *tetM*, *tetL* and *tetK* genes in *Lactococcus lactis*, *E. faecalis*, *Lactobacillus johnsonii* isolated from conventional cheese (Florez et al., 2014). Likewise, Yang & Yu. (2019) showed that *tetM* from *L. delbrueckii subsp. bulgaricus* R6 and *tetS* from *L. plantarum* R41 were successfully transferred to *L. monocytogenes* through filter mating. In contrast, some studies have revealed that LAB strains possess ARGs; however, their intrinsic resistance genes are not transferable to other LAB or pathogens through HGT (Anisimova et al., 2019; Guo et al., 2017). On the other hand, the absence of the tested ARGs in the enterocin-negative LAB isolates in the current study suggests their favorable safety and suitability for food application. Similar results have been reported by EFSA Panel on

FEEDAP et al. (2023), which demonstrated that *L. brevis* DSM 21982 does not carry acquired ARGs and is therefore considered safe for the target species, the environment, and consumers.

3.4.2. Screening for the virulence genes

In the current study, the assessment of the investigated virulence determinants, *hyl* and *esp* genes, indicated that out of the seven LAB strains tested, only one isolate, *L. paracasei* S8, was positive for the *hyl* gene, which was also positive for the *entAS-48* gene. However, the *esp* gene was not detected in any of the tested LAB isolates (Table 2). The *hyl* gene encodes hyaluronidase, an enzyme that degrades hyaluronic acid, a key structural component of the extracellular matrix in tissues, thereby facilitating bacterial dissemination (Kiruthiga et al., 2020). Although this enzyme can disrupt biofilms and enhance probiotic colonization, dysregulated hyaluronidase activity can result in host tissue damage and inflammatory responses (Kiruthiga et al., 2020). This gene is classically distributed among opportunistic and pathogenic bacteria, particularly within the genus *Enterococcus*, including *E. faecalis* and *E. faecium* (Jahansepa et al., 2022; Kiruthiga et al., 2020). As previously discussed, the presence of the *hyl* gene enterocin-positive *L. paracasei* S8 further supports the likelihood of HGT. The safety of LAB from artisanal dairy products has been extensively evaluated for the detection of virulence genes (Colautti et al., 2022; Dishan & Gönülalan, 2025; Zhao et al., 2022). However, studies reporting the presence of these genes are very scarce. The virulence-associated genes *hyl* and *esp* have been detected in *Latilactobacillus sakei* subsp. *sakei* 2a, highlighting the need for careful safety evaluation despite the strain's probiotic potential (Lipilkina et al., 2024). Despite its GRAS status and probiotic potential, the presence of the *hyl* gene in *L. paracasei* S8 raises potential safety concerns. Strains harboring both bacteriocin and virulence genes may be considered potentially unsafe, as they could pose risks to host health and may be unsuitable for technological applications. However, the absence of

both *hyl* and *esp* genes in the enterocin-negative LAB isolates indicates their biosafety profile and suitability for use in the food industry.

3.4.3. Detection of genes encoding BA-producing enzymes

BAs such as tyramine, histamine, putrescine, and cadaverine are produced by microbial decarboxylation of amino acids and are known to cause adverse health effects, ranging from food intolerance to toxicological reactions when present in high concentrations in fermented foods (Ladero et al., 2010a). These compounds can originate from raw milk, be produced by the starter culture or by secondary microbiota that develop during the fermentation process (Novella-Rodríguez et al., 2002). In this study, the isolated LAB strains were subjected to PCR for screening of genes encoding four key BA-producing enzymes: tyrosine decarboxylase, ornithine decarboxylase, histidine decarboxylase, and lysine decarboxylase. The results revealed their absence in all the tested *L. paracasei* and *Lev. brevis* strains. Several studies have reported that LAB from fermented foods are primary producers of BAs, with production often being strain-dependent rather than species-specific (Banicod et al., 2025; Barbieri et al., 2019; Ladero et al., 2015). In their study, Ladero et al. (2015) reported the presence of genes encoding BA-producing enzymes in *Lev. brevis* strains isolated from artisanal cheeses. In another study, tyramine-producing LAB such as *Lev. brevis*, *Lactobacillus curvatus* and *Enterococcus* spp., have been detected in all the analyzed milk and cheese samples (Ladero et al., 2010b). In the present study, the absence of genes encoding BA-producing enzymes in the tested LAB strains strongly supports their safety for food applications, as it indicates no risk of BA production. Their absence not only mitigates the risk of BA accumulation in fermented foods but also reinforces the candidacy of these LAB strains as promising natural bio-preservatives.

3.4.4. Detection of integron genes

The presence of integron genes in LAB may increase the risk of transmission of transferable ARGs to potential pathogenic bacteria and members of the host gut microbiota. Molecular characterization of integron genes demonstrated that *intI1* and *intI2* were not detected in any of the tested strains. Generally, these genes are predominantly associated with Gram-negative bacteria and are carried on mobile genetic elements such as transposons and plasmids (Li & Zhao, 2018; Faghri et al., 2018). These results may be explained by differences in the genomic and mobile-element architecture of LAB, which are less conducive to the integration and dissemination of typical Gram-negative-type integrons as previously suggested (Kaszab et al., 2023).

4. Conclusion

This is the first study reporting the detection of the *ent1071A/B* gene in non-enterococcal LAB, highlighting the expanded distribution of enterocin genes beyond the genus *Enterococcus*. Both enterocin-positive and -negative *Lactobacillus* spp. isolates displayed strong antibacterial activities against MDR foodborne pathogens. This study further provides a comprehensive safety assessment of LAB isolated from Algerian artisanal dairy products. Enterocin-negative LAB isolates lacked acquired ARGs, virulence determinants, biogenic amine-producing genes, and integron genes, supporting their suitability for food applications. However, the occurrence of *msrA* and *hyl* in enterocin-positive *L. paracasei* isolates emphasizes the importance of strain-level safety screening. Given the novelty of these findings, future studies should focus on whole-genome sequencing, plasmid analysis, and validation in food matrices to further elucidate the genetic context, transferability, and *in situ* expression of these determinants.

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.

Data availability statement

Data will be made available on request.

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Table 1. Antimicrobial resistance profile of the tested multi-drug resistant enterobacteriaceae isolates.

Strain	AMR profile	AMR genes	Reference
<i>E. coli</i> S55	AM, TIC, PIP, CZ, CF, CTX, NA, CIP, LVX, TE, SSS, TMP, SXT	<i>CTX-M-1, TEM</i>	Benamer et al. (2021)
<i>E. coli</i> S216	AM, AMC, TIC, PIP, CZ, CF, CTX, ATM, NA, TE, SSS	<i>CTX-M-1, qnrS1</i>	Benamer et al. (2018a)
<i>E. cloacae</i> S41	AM, AMC, PIP, CZ, NA, CIP, CS, TE, SSS, TMP, SXT, N		
<i>E. cloacae</i> S54	AM, AMC, PIP, CZ, NA, SXT, TE, N	<i>qnrS1</i>	Benamer et al. (2018b)
<i>K. pneumoniae</i> S20	AM, AMC, PIP, CZ, CF, CTX, NA, CIP, TE, SSS, TMP, SXT, N		
<i>S. typhimurium</i> S16	AM, AMC, PIP, CZ, CF, CTX, NA, CIP, TE, SSS, TMP, SXT, N		

E. coli: *Escherichia coli*; *E. cloacae*: *Enterobacter cloacae*; *K. pneumoniae*: *Klebsiella pneumoniae*; *S. typhimurium* : *Salmonella typhimurium*; AMR : antimicrobial resistance ; AM, ampicillin; AMC : ; TIC, ticarcillin; PIP, piperacillin; CZ, cefazolin; CF, cephalothin; CTX, cefotaxime; CAZ, ceftazidime; FEP: cefepime; ATM, aztreonam; CRO, ceftriaxone; NA, nalidixic acid; CIP, ciprofloxacin; LVX, levofloxacin; TE, tetracycline; SSS, sulfonamides; CT, colistin

Table 2. Phenotypic, physiological, and MALDI-TOF identification of Lactic acid bacteria isolated from Algerian artisanal dairy products.

Isolate code	Samples	Phenotypic identification					MALDI-TOF identification	Enterocin genes	Antimicrobial resistance determinants	Virulence genes
		Cell shape	Gram reaction	Catalase reaction	Oxidase	Motility				
S8	Fresh cream	Rod	+	-	-	-	<i>Lactocaseibacillus paracasei subsp. paracasei</i>	<i>entAS48</i>	/	<i>hyl</i>
S10	Butter	Rod	+	-	-	-	<i>Levilactobacillus brevis</i>	/	/	/
S43	Butter	Rod	+	-	-	-	<i>Levilactobacillus brevis</i>	/	/	/
S46	Butter	Rod	+	-	-	-	<i>Lactocaseibacillus paracasei subsp. paracasei</i>	/	/	/
S93	Lben	Rod	+	-	-	-	<i>Lactocaseibacillus paracasei subsp. paracasei</i>	<i>ent1071A/B</i>	<i>msrA</i>	/
S94	Lben	Rod	+	-	-	-	<i>Lactocaseibacillus paracasei subsp. paracasei</i>	/	/	/
S107	Lben	Rod	+	-	-	-	<i>Lactocaseibacillus paracasei subsp. paracasei</i>	/	/	/

(+): Positive reaction; (-): Negative reaction.

738 **Table 3.** Antibacterial potential of *Lactobacillus paracasei* and *Levilactobacillus brevis*
739 strains isolated from Algerian artisanal dairy products.

Indicator bacteria	<i>L. paracasei</i> S8	<i>Lev. brevis</i> S10	<i>Lev. brevis</i> S43	<i>L. paracasei</i> S46	<i>L. paracasei</i> S93	<i>L. paracasei</i> S94	<i>L. paracasei</i> S107	P
<i>E. coli</i> ATCC 25922	18.00±0.57	14.00±0.57 ^b	16.5±0.28 ^{ab}	15.5±0.28 ^{ab}	21.66±0.33 ^a	17.66±0.33 ^{ab}	13.66±0.33 ^b	0.0046
<i>K. aerogenes</i> ATCC 10006	16.0±0.57 ^b	14.66±0.33 ^{ab}	<6.0 ^a	14.0±0.0 ^{ab}	14.66±0.33 ^{ab}	16.66±0.33 ^b	13.66±0.33 ^{ab}	0.0068
<i>E. faecalis</i> ATCC 29212	22.0±0.57 ^{ab}	18.0±0.0 ^b	18.50±0.29 ^{ab}	19.0±0.0 ^{ab}	18.0±0.0 ^b	25.33±0.33 ^a	18.0±0.0 ^b	0.0043
<i>E. faecium</i> ATCC 6057	15.0±0.0 ^{ab}	15.5±0.28 ^{ab}	8.66±0.33 ^a	16.0±0.0 ^{ab}	16.0±0.0 ^{ab}	18.0±0.57 ^b	15.66±0.33 ^{ab}	0.0069
<i>P. aeruginosa</i> ATCC 27853	21.66±0.33 ^{ab}	17.50±0.29 ^{ab}	14.0±0.0 ^a	19.50±0.29 ^{ab}	20.33±0.67 ^{ab}	26.0±0.0 ^b	19.33±0.33 ^{ab}	0.0049
<i>S. aureus</i> ATCC 25923	26.0±0.57 ^{ab}	14.0±0.0 ^b	19.50±0.29 ^{ab}	25.0±0.0 ^{ab}	19.0±0.0 ^{ab}	18.5±0.28 ^{ab}	32.0±0.0 ^a	0.0036
<i>E. coli</i> S55	14.33±0.33 ^a	9.66±0.33 ^{ab}	11.66±0.33 ^{ab}	<6.0 ^b	11.0±0.0 ^{ab}	11.66±0.33 ^{ab}	11.66±0.33 ^{ab}	0.0063
<i>E. coli</i> S216	12.66±0.33 ^b	8.0±0.0 ^a	9.66±0.33 ^{ab}	12.66±0.33 ^b	11.0±0.0 ^{ab}	10.0±0.0 ^{ab}	10.0±0.0 ^{ab}	0.0037
<i>E. cloacae</i> S41	<6.0±0.0 ^a	14.33±0.33 ^b	13.66±0.33 ^{ab}	<6.0±0.0 ^a	12.66±0.33 ^{ab}	13.66±0.33 ^{ab}	14.0±0.0 ^b	0.0069
<i>E. cloacae</i> S54	12.66±0.33 ^{ab}	10.0±0.0 ^b	10.66±0.33 ^{ab}	10.66±0.33 ^{ab}	14.66±0.33 ^a	11.66±0.33 ^{ab}	10.66±0.33 ^{ab}	0.0091
<i>K. pneumoniae</i> S20	12.66±0.33	10.66±0.33	12.33±0.33	10.33±0.33	11.0±0.0	12.66±0.33	12.66±0.33	0.0111
<i>S. typhimurium</i> S16	<6.0 ^a	<6.0 ^a	<6.0 ^a	<6.0 ^a	13.0±0.0 ^b	<6.0 ^a	<6.0 ^a	0.0027
<i>S. enteritidis</i> S27	9.0±0.0 ^{ab}	<6.0 ^b	14.66±0.33 ^a	11.66±0.33 ^{ab}	12.0±0.0 ^{ab}	14.0±0.0 ^{ab}	14.0±0.57 ^{ab}	0.0043
<i>L. monocytogenes</i> S61	14.66±0.33 ^{ab}	16.33±0.33 ^b	11.0±0.57 ^a	14.33±0.33 ^{ab}	13.0±0.0 ^{ab}	13.0±0.33 ^{ab}	14.66±0.33 ^{ab}	0.0049

740 *L. paracasei*: *Lactobacillus paracasei* subsp. *paracasei*; *Lev. brevis*: *Levilactobacillus brevis*;
741 *E. coli*: *Escherichia coli*; *K. aerogenes*: *Klebsiella aerogenes*; *E. faecalis*: *Enterococcus*
742 *faecalis*; *E. faecium*: *Enterococcus faecium*; *P. aeruginosa*: *Pseudomonas aeruginosa*; *S.*
743 *aureus*: *Staphylococcus aureus*; *E. cloacae*: *Enterobacter cloacae*; *K. pneumoniae*:
744 *Klebsiella pneumoniae*; *S. typhimurium*: *Salmonella typhimurium*; *S. enteritidis*: *Salmonella*
745 *enteritidis*; *Listeria monocytogenes*: *L. monocytogenes*. ^{a/b/ab} Isolates with different letters are
746 significantly different from each other.

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General discussion

General discussion

The current study focused on the isolation and characterization of LAB from various Algerian artisanal dairy products. A total of twenty-nine (29) LAB strains were isolated from different fermented products, including Fresh cream, Buttermilk, and Lben. All of these products were originally prepared from raw cow's milk. Among the isolates, 11 strains were obtained from Fresh cream, 9 from Buttermilk, and 9 from Lben. Fermented dairy products are important ecological niches for LAB, providing favorable conditions that support their growth and adaptation (Amanullah *et al.*, 2021; Dosuky *et al.*, 2022; Roselli *et al.*, 2025). Interestingly, artisanal dairy products serve as valuable sources of LAB strains with potential probiotic properties, while also demonstrating significant variability linked to environmental conditions, regional production methods, and traditional knowledge (Leksir *et al.*, 2019; Benyahia *et al.*, 2025; Bouchibane *et al.*, 2025; Saci *et al.*, 2025;). Based on the MALDI-TOF identification, three species were detected: *Lev. brevis*, *L. paracasei*, and *E. faecalis*. The predominance of *L. paracasei* and *Lev. brevis* strains in dairy products contributes to their nutritional and functional qualities, thereby increasing consumer acceptance and demand.

Overall, these species are recognized as safe (GRAS) microorganisms (Kaur *et al.*, 2018; Ağagündüz *et al.*, 2021). Similarly, Arezki *et al.* (2020), Bentahar *et al.* 2024, and Bouchibane *et al.* (2022) reported the presence of these genera in numerous Algerian artisanal dairy products. Furthermore, these genera are generally associated with spontaneous fermentation, an artisanal process involved in the preparation of traditional dairy products such as *Jben*, *Lben*, *Klila*, *Raib*, *Butter milk*, and *Bouhezza*. However, *E. faecalis* is well known for its pathogenic potential, and its presence in food products may raise safety concerns (Gołaś-Prądyńska *et al.*, 2022). Moreover, the detection of *Enterococcus* spp. in dairy products is frequently associated with inadequate hygiene practices during production (Gajewska *et al.*, 2023). Biochemical and physiological characterization is an important approach to evaluate LAB strains under various stress conditions, including low pH, high salt concentrations, and a range of growth temperatures. Previous reports have indicated that temperature strongly influences the growth and metabolic activity of LAB (Yang *et al.*, 2018). Salt is widely used in the food industry not only as a flavor enhancer but also as a preservative due to its antimicrobial properties (Meng *et al.*, 2018; M'hamed *et al.*, 2022). Additionally, dairy products are considered reservoirs of technologically important LAB strains (Ledina *et al.*, 2024; Atter *et al.*, 2025). In the present study, most *Lev. brevis* and *L. paracasei* strains exhibited pronounced acidification and proteolytic activities, supporting their potential use as

probiotic additives or starter cultures in the dairy industry for the production of cheese and yogurt. The combination of *L. paracasei* and *Lev. brevis*, isolated from different dairy products, revealed significant potential for lactic acid production. This latter was shown to depend on several factors, including temperature, incubation period, pH, agitation, as well as inoculum age and inoculum volume (Duru *et al.*, 2025). On the other hand, *L. paracasei* strains studied demonstrated stronger proteolytic activity than *Lev. brevis* strains, consistent with recent study reporting similar findings (Cristofolini *et al.*, 2025). The observed technological performance can be attributed to the ability of LAB to hydrolyze casein into peptides and amino acids via specific proteolytic enzymes. These amino acids are subsequently utilized as a nitrogen source by LAB, as demonstrated by Ji *et al.* (2021). Furthermore, *Lactobacillus* spp. typically display greater proteolytic activity than *Lactococcus* or *Streptococcus* species (Kieliszek *et al.*, 2021). The proteolytic capacity of LAB varies widely depending on the species, subspecies, and strain. This property is important not only for technological characteristics such as improving sensory quality and generating functional peptides but also for human health, including reducing the allergenicity of milk proteins (Satılmış *et al.*, 2023).

Antimicrobial activity is an important criterion for probiotic selection (Choi *et al.*, 2018; Loforte *et al.*, 2025). In recent years, the widespread emergence and dissemination of MDR *Enterobacteriaceae* pose a significant global public health threat, due to limited treatment options and increased morbidity and mortality (Elshobary *et al.*, 2025; Furlan *et al.*, 2025). Additionally, the presence of these bacteria as spoilage organisms and foodborne pathogens in food systems represents a major challenge for the food industry, compromising food safety, shelf life, and consumer health (Mola *et al.*, 202; Amra *et al.*, 2024). In the current study, the majority of isolates were able to exhibit strong antagonistic effect, particularly against *E. coli* ATCC 25922, *P. aeruginosa* ATCC 27853, and *E. faecalis* ATCC 29212, indicating their ability to inhibit harmful pathogenic bacteria. This inhibitory effect is generally associated with the secretion of various postbiotics, including organic acids, hydrogen peroxide, and bacteriocins. Furthermore, the findings revealed moderate inhibitory activity against all tested MDR *Enterobacteriaceae* strains, such as *E. coli* S55, *E. coli* S216, *K. pneumoniae* 20. These results are consistent with previous studies reporting similar observations (Fijan *et al.*, 2018; Do *et al.*, 2024; Nada & Kotb, 2025). All *L. paracasei* and *Lev. brevis* strains carrying enterocin genes exhibited potent antimicrobial activity against the tested pathogenic microorganisms. In particular, *L. paracasei* S93 and *L. paracasei* S2, which harbor

ent1071A/B and *entQ* genes, respectively, were able to inhibit the growth of all the pathogens included in this study. These findings indicate that the presence of enterocin genes is strongly associated with enhanced antagonistic effect, supporting the potential use of these strains as effective probiotic or biocontrol agents against pathogenic bacteria.

The biosafety assessment showed that the majority of *L. paracasei* and *Lev. brevis* strains were characterized as safe, owing to the absence of integron genes, virulence genes, biogenic amine genes, and ARGs. This finding aligns with Chen et al. (2024), who reported the safety of the *L. paracasei* NTU 101 strain. However, the presence of ARGs and virulence genes in some LAB isolates may be explained by horizontal gene transfer, as previously demonstrated by Yang and Yu (2019), suggesting their transfer from *Enterococcus* spp., including *E. thailandicus* 52 to *E. faecalis* JH2-2 (Obioha et al., 2023). In this regard, it has been reported that LAB isolated from fermented dairy products can act as reservoirs of ARGs and may contribute to their horizontal transfer (Floris et al., 2025). Moreover, ARGs can be attributed to different genetic mechanisms. *Lactobacillus* genus exhibit intrinsic resistance to vancomycin due to structural modifications in their cell wall precursors, which result in reduced affinity for the antibiotic (Selim, 2021). The absence of the biogenic amine genes is considered a key marker of the safety of these strains. It is important to note that the ability to produce BAs is generally strain-specific (Colautti et al., 2024). The lack of these genes in the studied strains highlights their potential use in food applications as biopreservatives.

General conclusion

General conclusion

Algerian artisanal dairy products represent an important source of LAB with significant technological and probiotic potential. In this study, all LAB isolates were identified as *L. paracasei*, *Lev. brevis*, and *E. faecalis*, highlighting the microbial diversity naturally present in traditional fermented dairy products. The isolates demonstrated notable biotechnological properties, including strong acidification activity, antimicrobial effects, and, in some strains, lipolytic activity, which is particularly relevant for cheese production and flavor development. The antimicrobial activity exhibited by most LAB strains against a broad spectrum of pathogenic microorganisms, including MDR *Enterobacteriaceae*, fungal pathogens, and both Gram-positive and Gram-negative bacteria, underscores their potential role in improving food safety and shelf life. These effects are likely associated with the production of bioactive compounds such as organic acids, hydrogen peroxide, and bacteriocins.

To the best of our knowledge, molecular characterization of bacteriocin-encoding genes revealed, for the first time, the presence of enterocin genes (*entQ*, *entAS-48*, and *ent1071 A/B*) in some *L. paracasei* and *Lev. brevis* strains, suggesting possible horizontal gene transfer from *Enterococcus* species. This new report supports their application in biopreservation.

Safety assessment revealed the absence of biogenic amine-producing and integron-associated genes in all isolates. However, the presence of virulence factors (*hyl* and *esp*) in some strains, as well as the detection of *msrA* in *L. paracasei* S93, raises significant safety concerns. These findings indicate that the affected strains are unsuitable for use in food or health-related applications. In contrast, other *L. paracasei* and *Lev. brevis* strains lacking virulence and resistance determinants exhibited a favorable biosafety profile, supporting their potential use as safe probiotic candidates. In general, this study emphasizes the importance of selecting and evaluating the safety of LAB strains for industrial and therapeutic applications. The use of safe probiotic strains from artisanal dairy products represents a promising alternative to antibiotics and may contribute to addressing the global challenge of antibiotic resistance.

For future perspectives, it will be necessary to continue this research. The following actions are recommended:

- ✓ Whole-genome sequencing of *L. paracasei* and *Lev. brevis* strains harboring enterocin genes should be performed to confirm gene functionality, genetic stability, and safety.
- ✓ Investigation of potential synergistic effects between probiotic LAB strains and essential oils, as well as interactions with other beneficial microorganisms isolated from different sources.
- ✓ Evaluation of the selected LAB strains as starter or adjunct cultures in dairy product manufacturing to assess their impact on product quality, safety, and sensory properties
- ✓ Exploration of the use of these strains in agriculture to promote plant growth and as environmentally friendly biofertilizers.
- ✓ Further evaluation of additional bioactivities, including antioxidant, antibacterial, and anti-inflammatory properties.
- ✓ Identification and characterization of the antimicrobial compounds responsible for the observed antagonistic activity to support their practical application.
- ✓ *In vivo* studies to assess the probiotic potential of selected strains and their possible use as alternatives to antibiotics.

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Scientific communications

International Conference Communications

1. **Roumaissaa BELKACEM, Qada BENAMEUR, Hajer KILANI.** Biodiversity of lactic acid bacteria isolated from Algerian artisanal dairy products. Mediterranean Conference on Environement Risks and Health (MCERH'2024). Faculty of Natural and Life Sciences, University of Mohamed Cherif Messaadia, Souk Ahras, Algeria.
2. **Roumaissaa BELKACEM, Qada BENAMEUR, Hajer KILANI.** Antimicrobial Potential of Some Probiotic Strains Isolated From Artisanal Dairy Products Againts Multidreug Resistant Enterobacteriaceae. Beyond Sciences Initiative 10th International Remote Conference: Science & Sociaty, poster presentation. Beyond Science Initative, Canada.
3. **Roumaissaa BELKACEM, Qada BENAMEUR, Hajer KILANI.** Molecular characterization of bacteriocin-encoding genes in lactic acid bacteria isolated from artisanal dairy products. The First Virtual International Congress on Computational Biology. Bioinformatics and Artificial Intelligence (1st VIC- CBBAI- 25). Faculty of Natural and Life Sciences, Saad Dahlab University of Blida 1, Blida, Algeria.

National conference communication

1. **Roumaissaa BELKACEM, Qada BENAMEUR.** Penotypic and Technological Characterization of Lactic Acid Bacteria From Artisanal Dairy Products of Mostaganem Province. 1st National Forum on Bioscience, Biotechnologies and Biodiversity (NFBBB '24). Faculty of Natural and Life Science, Hassiba Benbouali University of Chlef.