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Article

Lactic Acid Bacteria from Traditional Fermented Milk: Antimicrobial Potential Against Foodborne Pathogens

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Abstract

Lactic acid bacteria (LAB) are increasingly recognized for their role in food biopreservation due to their ability to synthesize antimicrobial compounds. Milk naturally harbors a wide variety of lactic acid bacteria, offering a promising source for identifying strains with biopreservative potential. This study investigated the antagonistic effects, safety characteristics, and technological properties of LAB strains isolated from traditionally fermented milk. Thirty-two dairy samples were analyzed, and the resulting LAB isolates were screened for inhibitory activity against *Listeria monocytogenes* CECT 4032 and *Staphylococcus aureus* CECT 976 using agar spot and well diffusion assays. All tested strains exhibited strong antimicrobial effects, with particularly notable inhibition of *L. monocytogenes*. After phenotypic screening, five representative isolates were selected for molecular identification and further assessment of safety-related attributes, functional capabilities, auto- and co-aggregation properties. 16S rRNA gene sequencing revealed that four strains belonged to the genus *Enterococcus*, specifically, one *E. faecium* and three *E. durans*, while one was classified as a *Lactococcus* species. Moreover, none of the strains showed proteolytic or lipolytic activities which highlights their potential use in dairy fermentation processes.

Keywords: fermented milk; Bacteriocin-like activity; *Lactococcus*; *Enterococcus*; foodborne pathogens

1. Introduction

Lactic acid bacteria (LAB) have attracted considerable scientific interest due to their capacity to produce a broad spectrum of antimicrobial substances. These metabolites contribute to the inhibition of undesirable microorganisms, such as spoilage bacteria and foodborne pathogens, thereby enhancing the microbial safety and extending the shelf life of a wide range of food products. Their natural occurrence in fermented foods and recognized safety status further support their application as bio-preservative agents in food systems [1]. These Gram-positive, fermentative bacteria are classified within the phylum Firmicutes, which includes a variety of genera important in food fermentation [2]. The incorporation of LAB into food systems contributes significantly to improving sensory qualities, including flavor, consistency, and nutritional content. Beyond their well-known technological contributions to fermentation, LAB also play a pivotal role in improving food safety. This is largely due to their capacity to inhibit the growth of spoilage microorganisms and pathogenic bacteria through the synthesis of various antimicrobial compounds. By producing such bioactive metabolites, LAB help create an inhospitable environment for undesirable microbes, thereby contributing to the microbiological stability and safety of food products [3,4]. Lactic acid bacteria exhibit antimicrobial activity through the synthesis of various bioactive metabolites. Among the antimicrobial compounds produced by LAB, organic acids, particularly lactic and acetic acid, play a fundamental role in pathogen inhibition. These acids reduce the pH of the surrounding environment,

thereby creating conditions that are hostile to the survival and proliferation of many spoilage and pathogenic microorganisms. In addition to organic acids, LAB produce a variety of other antimicrobial substances that contribute to their protective role in food systems. These include diacetyl, which interferes with bacterial metabolism; carbon dioxide (CO₂), which exerts an inhibitory effect by reducing oxygen availability; and hydrogen peroxide, which induces oxidative stress in microbial cells. Moreover, LAB are known for their ability to synthesize bacteriocins, ribosomally produced antimicrobial peptides that exhibit targeted activity against closely related bacteria, including several foodborne pathogens. These compounds often act synergistically, enhancing the overall inhibitory effect against a broad spectrum of spoilage and pathogenic microorganisms. The combined action of these compounds enhances the overall inhibitory potential of LAB in fermented food systems [5,6]. Among the various antimicrobial substances produced by LAB, bacteriocins are considered especially promising. These peptides exhibit strong inhibitory effects against a wide range of pathogenic bacteria associated with foodborne illnesses. Their potent antimicrobial properties make them promising agents for improving food safety and prolonging product shelf life [7,8]. *Lactococcus* and *Enterococcus* species are commonly found in raw and fermented milk, reflecting their strong adaptation to dairy ecosystems [9,10]. Species such as *L. garvieae* and various subspecies of *L. lactis* have been reported to produce a broad spectrum of bacteriocins [11]. Notably, *L. lactis* strains exhibiting antimicrobial activity have gained considerable interest as promising biopreservatives in the dairy industry [12]. *Enterococcus* species likewise produce enterocins, a group of bacteriocins that exhibit antimicrobial activity against Gram-positive bacteria, including important foodborne pathogens like *Listeria* spp. [13]. Among *Enterococcus* species, *E. faecalis* and *E. faecium* are the main producers of enterocins. These strains have been extensively studied owing to their reliable ability to generate various types of enterocins with strong antimicrobial effects [14]. Numerous studies have focused on isolating LAB with antimicrobial activity from dairy matrices. For instance, Achemchem et al. [15] reported the isolation of the bacteriocin-producing *Enterococcus faecium* F58 strain from traditional soft goat cheese, while Ghrairi et al. [16] reported LAB with anti-*Listeria* activity from Rigouta, a traditional Tunisian cheese. Additionally, Perin, and Nero [17] identified *Lactococcus* and *Enterococcus* as predominant genera in the microbial community of raw goat's milk. They also highlighted the ability of these bacteria to inhibit the growth of *Listeria monocytogenes* ATCC 7644. Elotmani et al. [18] also reported the presence of LAB with antilisterial activity in Raïb, a traditional Moroccan fermented milk. In light of these findings, the present study aimed to isolate LAB strains from traditionally fermented milk and assess their antimicrobial activity against key foodborne pathogens. Special emphasis was placed on evaluating the ability of these isolates to produce bacteriocins, antimicrobial peptides known for their targeted inhibitory effects against pathogenic and spoilage microorganisms. Additionally, their safety characteristics and technological functionalities were examined to determine their suitability for application in cheese biopreservation.

2. Materials and Methods

2.1. Isolation of LAB from Milk

A total of 32 samples of spontaneously fermented milk were analyzed to isolate LAB. Samples were serially diluted in sterile saline to obtain appropriate concentrations for microbial plating and then applied onto two selective culture media, Man, Rogosa, and Sharpe (MRS) agar (Biokar Diagnostics, France) and M17 agar (Biokar Diagnostics, France) supplemented with 0.5% glucose (GM17). The inoculated plates were incubated at 30°C for 24 to 48 hours. After incubation, colonies showing typical LAB morphology were isolated and purified. The bacterial isolates obtained were subsequently evaluated for their ability to inhibit the growth of specific pathogenic bacteria.

2.2. Screening for Bacteriocinogenic LAB

To screen for bacteriocin production, several colonies were randomly picked and transferred onto two sets of agar plates for further analysis [15]. These plates were then incubated anaerobically

at 30°C for 16 to 24 hours to allow sufficient bacterial growth. After the initial incubation period, the plates were overlaid with a layer of soft agar (6ml, 0.75% agar) that had been inoculated with approximately 10⁸ CFU/mL of an overnight culture of the indicator organisms, either *Listeria monocytogenes* CECT 4032 or *Staphylococcus aureus* 976. These indicator strains were previously cultivated in Tryptic Soy Broth (TSB) (Biokar Diagnostics, France) under optimal conditions to ensure sufficient growth and viability before use in the overlay assay. The formation of clear inhibition zones indicated antimicrobial activity.

2.3. Indicator Pathogens and Antimicrobial Spectrum

The inhibitory potential of the isolated strains was assessed using both the agar spot and well diffusion methods, following the methods described by Achemchem et al. [19]. In the agar spot test, 5 µL of overnight LAB cultures grown in either MRS or GM17 broth were carefully spotted onto the surface of solidified agar plates of the corresponding medium. After incubation at 30 °C for 24 hours to allow bacterial growth, The plates were overlaid with soft agar previously inoculated with an overnight culture of the indicator organisms. The overlay was poured gently to ensure even distribution across the plate surface. Following a second incubation, inhibition zones around the colonies were observed as indicators of antimicrobial activity.

For the well diffusion assay, LAB strains were cultured overnight at 30°C in MRS or GM17 broth. The cultures were then centrifuged, and the resulting supernatants were collected to obtain cell-free supernatants (CFS). Wells were created in Mueller Hinton Agar (MHA) plates (Biokar Diagnostics, France) using sterile stainless-steel cylinders. After wells were prepared in the agar medium, each plate was overlaid with soft agar previously inoculated with an overnight culture of the designated indicator microorganism. Once the overlay solidified at room temperature, 100 µL of the prepared CFS was carefully added to each well. To ensure uniform diffusion of antimicrobial compounds into the agar, the plates were first incubated at 4 °C for 2 hours. This was followed by incubation at 37 °C for 16 hours to allow the growth of the indicator strain and expression of any inhibitory activity. Antimicrobial effects were assessed by measuring the diameter of the clear inhibition zones around the wells.

Ten pathogenic bacterial strains were chosen as indicator organisms to assess the antimicrobial activity of the tested isolates. The selected Gram-positive strains included *L. monocytogenes* CECT 4032, CECT 7467, CECT 5725, and CECT 935; *S. aureus* CECT 976; and *Bacillus subtilis* DSMZ 6633. The Gram-negative group consisted of *Pseudomonas aeruginosa* CECT 118, *Escherichia coli* ATCC 25922 and CECT 4076, and *Salmonella enterica* CECT 704. All indicator strains were cultivated in TSB at 37°C for 18 hours before use in the assays. As a positive control, *Enterococcus faecium* F58, a well-characterized bacteriocin-producing strain, was grown on MRS agar under standard incubation conditions.

2.4. Stability of Bacteriocin-like Activity After Exposure to Proteolytic Enzymes

To evaluate whether the antibacterial compounds produced by the isolated strains were proteinaceous, their sensitivity to proteolytic enzymes was tested. Cell-free supernatants (CFS) obtained from cultures at the early stationary phase were incubated at 37 °C for 2 hours with proteinase K (1 mg/mL), prepared in the buffer recommended by the manufacturer (Qiagen, Germany). *L. monocytogenes* CECT 4032 was used as the indicator strain, while untreated CFS served as negative controls. Residual antibacterial activity in both treated and untreated samples was assessed through the well diffusion assay.

2.5. Identification of Antagonistic LAB

2.5.1. Phenotypic and Biochemical Identification

Strains producing bacteriocins were first examined using phenotypic methods, including Gram staining, catalase activity testing, and microscopic observation of cellular morphology. Their

biochemical characteristics were also evaluated, such as their ability to grow at 10 °C and 45 °C, tolerate 4% and 6.5% NaCl, and grow on Bile Esculin Azide (BEA) agar (Biokar Diagnostics, France). To achieve more precise taxonomic identification, the isolates were analyzed using the API 20 Strep identification system (bioMérieux, France), according to the manufacturer's guidelines.

2.5.2. Molecular Identification (16S rRNA)

Following the procedure described by Elidrissi et al. [20], genomic DNA was extracted from bacterial cultures and quantified. A fragment of about 1500 base pairs from the 16S rRNA gene was amplified using universal primers in a 25 µL PCR reaction, with standard thermal cycling including an initial denaturation, 35 cycles of denaturation, annealing, extension, and a final elongation step. The PCR products were checked by agarose gel electrophoresis. Purified amplicons were then sequenced using specific primers. The sequencing products were further purified, analyzed by capillary electrophoresis, and the resulting sequences were identified by BLAST comparison against the NCBI database.

These procedures were performed at the Technical Support Units for Scientific Research (UATRS), affiliated with the National Center for Scientific and Technical Research (CNRST), Morocco.

2.6. Technological Assessment of LAB Strains

2.6.1. Proteolytic and Lipolytic Activities

Proteolytic activity was evaluated by inoculating the isolates onto Trypticase Soy Agar (TSA) (Biokar Diagnostics, France) containing 1.5% (w/v) skim milk, following the protocol of Achemchem et al. [19]. The presence of clear halos around the colonies was used as an indicator of proteolytic activity.

The lipolytic activity of the isolates was examined by testing them on nutrient agar enriched with 10 g/L Tween 20 and 0.1 g/L calcium chloride, following the protocol established by Albayrak, and Duran [21]. Ten microliters of each culture were placed onto the prepared medium and incubated under anaerobic conditions at 37 °C for 72 hours. The detection of lipase production was based on the visual observation of precipitate formation due to the interaction between calcium ions and fatty acids.

2.6.2. Aggregation Abilities

The aggregation properties of the selected LAB strains were evaluated to determine their potential for adhesion and interaction with pathogenic microorganisms, following the procedures outlined by Elidrissi et al. [20] and Zanzan et al. [22].

Auto-aggregation tests were conducted to evaluate the ability of LAB strains to adhere to their own cells. The degree of auto-aggregation was calculated as a percentage using the formula: Auto-aggregation (%): $1 - (A_t/A_0) \times 100$, where A_0 and A_t represent the initial and final absorbance at 600 nm, respectively.

To determine the co-aggregation capacity between LAB strains and the foodborne pathogen *L. monocytogenes* CECT 4032, equal volumes of each bacterial culture were combined and incubated at room temperature without agitation for 4 and 24 hours. Absorbance measurements were taken for the individual cultures as well as their mixture. Co-aggregation percentage was calculated using: Coaggregation % = $((A_{lab} + A_{pat}) - A_{mix}) / (A_{lab} + A_{pat}) \times 100$, where $A_{lab} + A_{pat}$ are the initial absorbances of the LAB and pathogen suspensions, respectively, and A_{mix} is the absorbance of the mixture at the specified time point.

2.7. Evaluation of Safety-Related Traits

2.7.1. Hemolytic Activity

The bacterial isolates were tested for hemolytic activity by inoculating them onto blood agar containing 5% sheep blood, followed by incubation at 37 °C for 48 hours. After incubation, the presence of hemolysis was evaluated [23]. *S. aureus* CECT 976 served as the positive control.

2.7.2. Antibiotic Susceptibility

The antibiotic susceptibility of the bacterial isolates was evaluated using the disc diffusion method, following the procedure described by Doménech-Sánchez et al. [24]. All isolates were tested against a range of antibiotics.

The antibiotics tested in this research comprised streptomycin (25 µg), vancomycin (30 µg), netilmicin (30 µg), ampicillin (20 µg), ciprofloxacin (5 µg), penicillin G (10 units), erythromycin (15 µg), chloramphenicol (30 µg), fosfomicin (200 µg), fusidic acid (10 µg), tetracycline (30 µg), gentamicin (10 µg), and kanamycin (30 µg). The inhibition zone diameters around the antibiotic discs were measured, and the results were interpreted based on the guidelines provided by CLSI [25] and Mazlumi et al. [26]. Based on the inhibition zone diameters, isolates were classified as susceptible, intermediate, or resistant.

2.7.3. Gelatinase activity and Biogenic Amine Production

Gelatinase activity was assessed by inoculating 1 µL of an overnight culture onto Brain Heart Infusion (BHI) agar (Biokar Diagnostics, France) supplemented with 3% (v/v) gelatin, following the protocol described by Amidi-Fazli, and Hanifian [27]. The inoculated plates were incubated at 37 °C for 48 to 72 hours, then transferred to 4 °C for 4 hours. The appearance of opaque halos surrounding the colonies was interpreted as a positive indication of gelatinase activity.

The ability of the LAB isolates to produce biogenic amines was assessed using a decarboxylase assay, following the method described Majjala [28]. The test was carried out on Majjala agar medium supplemented with 20 g/L histidine, used as the amino acid pre-cursor. The formation of a purple coloration in and around the bacterial colonies served as an indicator of histamine production.

2.9. Statistical Analysis

Statistical analyses were performed using one-way analysis of variance (ANOVA), followed by Tukey's post hoc test, with Statistica version 6.0.

3. Results

3.1. Bacteriocinogenic Potential of LAB Strains

Lactic acid bacteria isolated from dairy-derived samples were subjected to initial screening to assess their antimicrobial potential. This preliminary evaluation employed the double-layer agar technique against two common foodborne pathogens: *L. monocytogenes* CECT 4032 and *S. aureus* CECT 976. Isolates that demonstrated inhibitory effects against at least one of these indicator strains were subsequently selected for further analysis. Their capacity to produce antimicrobial metabolites was then investigated using the agar well diffusion method. To confirm these findings, the antagonistic activity of the isolates was also tested in liquid culture against both *L. monocytogenes* and *S. aureus* (Table 1).

Out of all the isolates tested, 18 LAB strains demonstrated significant antimicrobial activity, producing distinct inhibition zones ranging from 11 to 19 mm in diameter against one or both target pathogens. These strains were subjected to basic phenotypic characterization, including Gram staining, catalase testing, and assessment of growth under various environmental conditions (Table

1). All selected isolates were Gram-positive, catalase-negative, and exhibited coccoid morphology under microscopic observation, features characteristic of typical LAB.

To further characterize these antimicrobial strains, the API 20 STREP identification system was used. From this group, five representative isolates were selected for molecular identification by 16S rRNA gene sequencing. Sequence alignment revealed a high degree of similarity to known LAB reference strains available in the GenBank database (Table 2). The phylogenetic relationships of these isolates are depicted in Figure 1, highlighting their clustering with closely related species. Based on the 16S rRNA sequence data, the strains were identified as *Lactococcus lactis*, *Enterococcus faecium*, and *Enterococcus durans*.

Eighteen strains were assessed for their ability to inhibit different pathogenic microorganisms. Using the spot method, all tested LAB isolates demonstrated clear antimicrobial activity against *Listeria* spp., indicating a strong antagonistic potential. In addition to this consistent antilisterial effect, several strains exhibited inhibitory activity against a broader panel of pathogens, including *S. aureus* CECT 976, *Bacillus subtilis* DSMZ 6633, *Escherichia coli* ATCC 25922, *E. coli* CECT 4076, *Salmonella enterica* CECT 704, and *Pseudomonas aeruginosa* CECT 118 (Tables A1 and A2).

In the agar well diffusion assay, all selected LAB strains exhibited clear inhibitory effects against *L. monocytogenes*, confirming their ability to produce antimicrobial substances capable of diffusing through the agar medium. No inhibition zones were detected against either of the *E. coli* strains or *S. enterica* CECT 704, indicating the absence of measurable activity against these Gram-negative bacteria under the tested conditions.

Strain-specific antimicrobial patterns were observed against other indicator organisms. *E. faecium* KB1 showed inhibitory activity against *P. aeruginosa* CECT 118. *E. durans* strains KB3, KB10, and KB13 inhibited *B. subtilis* DSMZ 6633, while *L. lactis* KB14 exhibited activity against *S. aureus* CECT 976. These results highlight the variability in antimicrobial performance among the tested LAB strains, with some demonstrating broader spectra of activity beyond *L. monocytogenes*.

The cell-free supernatants from the five selected strains were treated with proteinase K to assess the nature of the antimicrobial compounds. The enzymatic digestion neutralized the inhibitory activity, indicating that the active substances were proteinaceous, likely corresponding to bacteriocins (Figure 2).

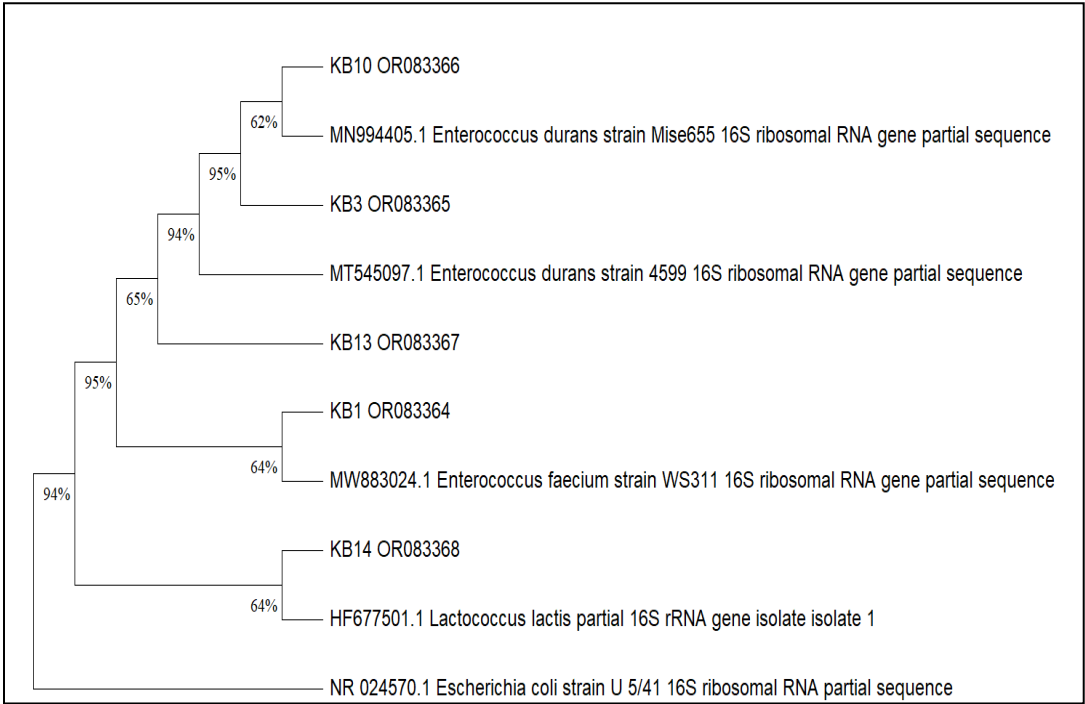


Figure 1. Phylogenetic Tree of Selected LAB Strains.

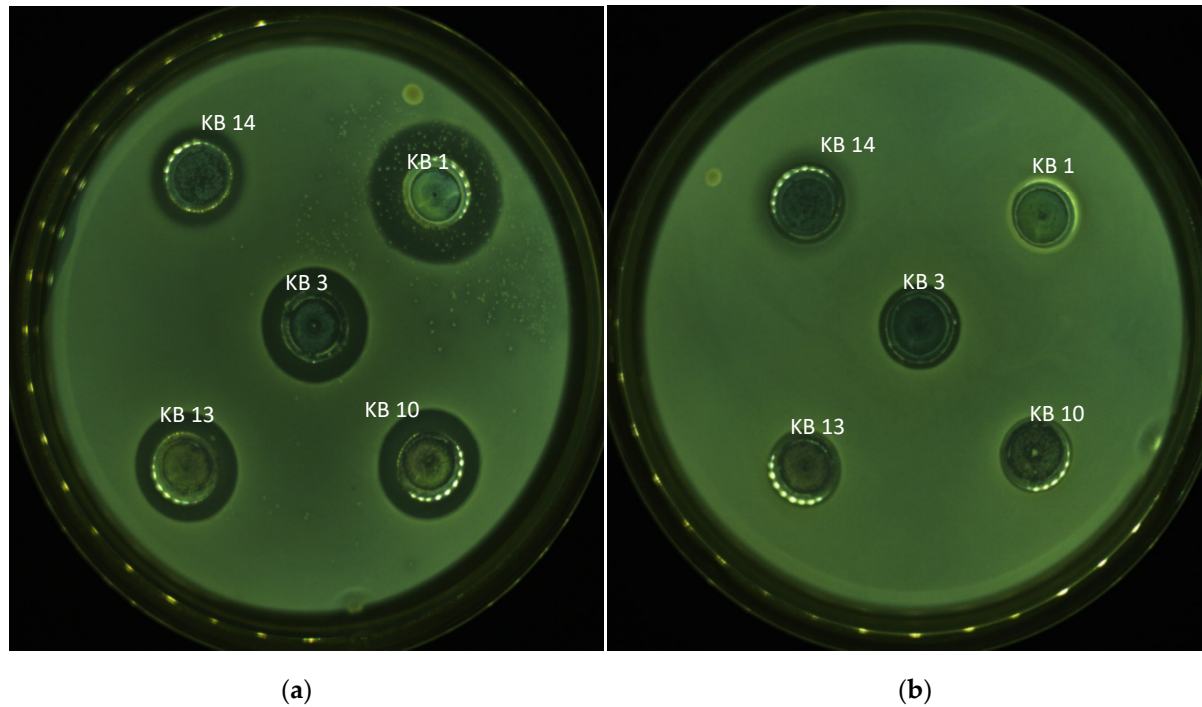


Figure 2. Impact of Proteinase K Treatment on the Antibacterial Activity of Selected Strains: (a) Untreated Control; (b) Treated with Proteinase K.

Table 1. Phenotypic Identification of Selected Strains of LAB.

Strains	Gram Staining	Catalase Activity	Growth Ability at 10°C	Growth Ability at 45°C	Growth Ability in 4% NaCl	Growth Ability in 6,5%NaCl	Growth in Bile-Esculin-Azide Agar
KB1	+	-	+	+	+	+	+
KB2	+	-	+	+	+	+	+
KB3	+	-	+	+	+	+	+
KB4	+	-	-	+	+	+	+
KB5	+	-	+	+	+	+	+
KB6	+	-	+	+	+	+	+
KB7	+	-	+	+	+	+	+

KB8	+	-	+	+	+	+	+
KB9	+	-	+	+	+	+	+
KB10	+	-	+	+	+	+	+
KB11	+	-	+	+	+	+	+
KB12	+	-	+	+	+	+	+
KB13	+	-	+	+	+	+	+
KB14	+	-	+	+	+	+	+
KB15	+	-	+	+	+	+	+
KB16	+	-	+	+	+	+	+
KB17	+	-	+	+	+	+	+
KB18	+	-	+	+	+	+	+

+: Positive reaction or Growth; -: Negative reaction or Lack of growth.

Table 2. Genetic Identification of Selected Antibacterial LAB Strains.

Selected strains	Origin of isolate	Identification	Number of accessions
KB1	Goat's milk	<i>E. faecium</i>	OR083364
KB3		<i>E. durans</i>	OR083365
KB10		<i>E. durans</i>	OR083366
KB13		<i>E. durans</i>	OR083367
KB14		<i>L. lactis</i>	OR083368

None of the five tested strains exhibited gelatinase or hemolytic activity, indicating the absence of these potential virulence factors. Table 3 summarizes the antibiotic susceptibility results for the five isolates evaluated in this research. All strains were susceptible to fosfomycin and tetracycline. However, strain KB1 exhibited resistance to penicillin G, streptomycin, gentamicin, netilmicin, and kanamycin. Strain KB3 showed resistance only to streptomycin, while KB13 was the only isolate resistant to chloramphenicol. In contrast, strains KB14 and KB10 exhibited favorable susceptibility profiles, being either sensitive or intermediately susceptible to all 13 antibiotics tested. Additionally, none of the isolates showed proteolytic or lipolytic activity, further supporting their safety potential for use in food-related applications.

Table 3. Antibiotic Susceptibility of Selected LAB Strains.

Antibiotic	KB1	KB3	KB10	KB13	KB14
Vancomycin	I	S	S	I	S
Fosfomycin	S	S	S	S	S
Penicillin G	R	S	S	S	S
Ampicillin	I	S	S	S	S
Ciprofloxacin	I	S	I	I	I
Fusidic Acid	I	S	S	S	I
Streptomycin	R	R	I	I	I
Gentamicin	R	S	I	I	I
Chloramphenicol	S	S	S	R	S
Netilmicin	R	S	I	I	I
Erythromycin	R	S	S	S	S
Tetracycline	S	S	S	S	S
Kanamycin	R	S	I	I	I

3.2. Aggregation Capacity of LAB Isolates

The ability of the five selected LAB isolates to auto-aggregate was evaluated over time. After 4 hours of incubation, auto-aggregation levels varied considerably among the strains, ranging from $0.35 \pm 0.50\%$ to $27.53 \pm 6.46\%$ (Figure 3). A substantial increase was observed after 24 hours, with values reaching between $28.21 \pm 7.25\%$ and $59.56 \pm 1.48\%$, indicating a time-dependent enhancement in cell-to-cell aggregation capacity. This characteristic is often associated with improved colonization potential and stability in microbial communities.

Co-aggregation with *L. monocytogenes* CECT 4032 also revealed strain-specific differences (Figure 4). Among the tested isolates, *L. lactis* KB14 showed the lowest level of co-aggregation, while *E. durans* KB3 exhibited the highest interaction with the pathogen. After 24 hours, co-aggregation values ranged from $30.00 \pm 0.38\%$ to $55.52 \pm 5.96\%$, with statistically significant differences between strains ($p < 0.05$). These findings suggest varying potential among isolates for pathogen exclusion through co-aggregation, which may contribute to their probiotic or bioprotective roles.

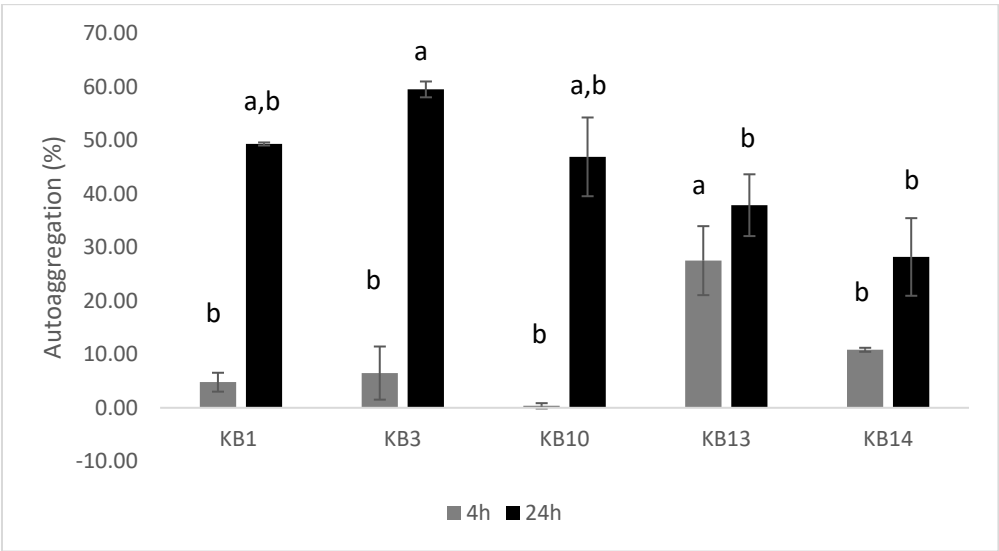


Figure 3. Auto-aggregation Ability (%) of LAB Isolated from Fermented Goat’s Milk.

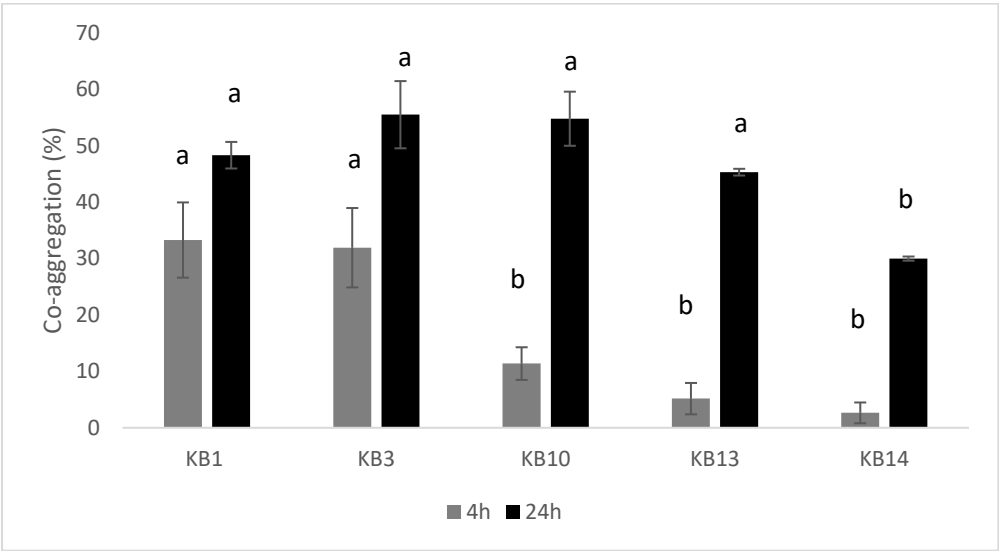


Figure 4. Co-aggregation Ability (%) of LAB Isolated from Fermented Goat's Milk.

4. Discussion

4.1. Antimicrobial Potential and Safety Assessment of LAB Strains

In the present study, LAB isolated from raw goat’s milk exhibited pronounced antimicrobial activity against *L. monocytogenes*, corroborating previous findings that emphasize the inhibitory potential of dairy-derived LAB [6,15,17,29–33]. Prior research has particularly highlighted the antimicrobial capabilities of *E. faecium*, *E. durans*, and *L. lactis*, especially those isolated from traditional artisanal cheeses. These strains have demonstrated the ability to inhibit not only *L. monocytogenes*, but also a range of other foodborne pathogens, including *Bacillus cereus* and *P. aeruginosa* [15,34–40].

Antibiotic susceptibility testing revealed that none of the isolates exhibited resistance to vancomycin, in agreement with previously published data [21,40,41]. Notably, *L. lactis* KB14, isolated in this study, was susceptible to ampicillin, chloramphenicol, and erythromycin, in line with the findings reported by Kazancigil et al. [42].

Collectively, these findings support the potential application of the studied LAB strains as natural biopreservatives in cheese production. Their strong antimicrobial activity and favorable

antibiotic resistance profiles suggest that they could contribute to enhancing food safety and extending shelf life in fermented dairy products.

4.2. Proteolytic and Lipolytic Activities

Our results are consistent with those of Albayrak, and Duran [21], who reported that *Enterococcus* strains isolated from dairy sources lacked proteolytic activity. This absence of proteolytic function suggests that certain *Enterococcus* spp. may have limited capacity to degrade milk proteins, potentially preserving the structural integrity and sensory attributes of fermented dairy products. However, proteolytic activity is not universally absent in this genus. Several other studies have reported the presence of proteolytic enzymes in *Enterococcus* species, highlighting strain-dependent variability. For instance, Achemchem et al. [19], de Sousa et al. [38], Mercha et al. [43], and Islam et al. [44] observed proteolytic activity in different *Enterococcus* strains, suggesting that some isolates may contribute to flavor development or textural changes.

Regarding *Lactococcus* strains, similar variability in proteolytic capabilities has been noted. With regard to *Lactococcus* strains, Cheng et al. [45], reported that the isolates tested exhibited proteolytic activity. Conversely, Allam et al. [46] identified two *Lactococcus* strains that lacked this enzymatic function, reinforcing the notion that proteolytic capacity is strain-specific and must be evaluated individually when selecting candidates for starter or adjunct cultures.

In our study, none of the tested strains displayed lipolytic activity, as no visible hydrolysis of lipids was observed. This finding aligns with previous reports indicating that nisin-producing *L. lactis* strains generally lack lipolytic enzymes [12]. Similarly, multifunctional LAB strains isolated from artisanal cheeses have also been shown to be non-lipolytic [21]. The absence of lipolytic activity is particularly advantageous in dairy fermentation, as excessive lipid hydrolysis can lead to off-flavors or rancid notes, compromising product quality.

Taken together, these results suggest that the selected LAB strains, due to their lack of both proteolytic and lipolytic activities, may be suitable for applications in dairy products where minimal enzymatic degradation is desired. Their neutral enzymatic profile makes them excellent candidates for preserving the sensory integrity of fresh cheeses or for use as protective cultures in biopreservation strategies without negatively influencing flavor or texture.

4.3. Auto-Aggregation and Co-Aggregation Capabilities of LAB Strains

The capacity of LAB strains to auto-aggregate and co-aggregate is widely recognized as a key functional characteristic that underpins their ability to persist and colonize host environments, such as the gastrointestinal tract. These aggregation properties contribute not only to microbial adhesion and biofilm formation but also to the exclusion of potential pathogens through competitive mechanisms. In the present study, the evaluated LAB strains exhibited varying levels of auto-aggregation after 4 hours of incubation, with some isolates demonstrating significantly stronger aggregation capabilities than others. These results align with those reported in the literature [42,47,48]. A longer incubation period (24 hours) led to a noticeable enhancement in auto-aggregation capacity for all tested strains, particularly for *E. durans* KB3, which exhibited the highest level of auto-aggregation. This increase over time may reflect enhanced expression of surface proteins or exopolysaccharides that promote cell–cell adhesion during prolonged growth phases.

In terms of co-aggregation, the tested strains also displayed a time-dependent increase in their ability to aggregate with *L. monocytogenes* CECT 4032, in agreement with patterns previously observed in the literature [49,50]. Notably, the co-aggregation ability of the *Lactococcus* strain was significantly lower than that of *Enterococcus* strains after 24 hours of incubation, suggesting species-specific differences in surface structures or in the composition of extracellular polymeric substances involved in cell interactions.

Both auto- and co-aggregation are considered beneficial traits for probiotic and bioprotective functions. Auto-aggregation is often associated with enhanced biofilm-forming ability, which facilitates stronger adhesion to intestinal epithelial surfaces and promotes colonization. Co-

aggregation, on the other hand, plays a crucial role in the exclusion of enteric pathogens by forming physical barriers or interfering with pathogen adhesion, thus contributing to microbial equilibrium and host protection [33,51]. Collectively, these characteristics underline the promising potential of the selected LAB strains not only as probiotics capable of colonizing and persisting in the gastrointestinal environment but also as effective functional cultures for application in food systems, particularly for improving microbial safety and shelf life.

4.4. Protein-Based Features of Antimicrobial Substances from LAB

The LAB strains evaluated exhibited antimicrobial activity suggestive of bacteriocin production. These inhibitory effects were eliminated following treatment with a proteolytic enzyme (Figure 2), confirming the proteinaceous nature of the active compounds. Bacteriocin-producing LAB are increasingly recognized as promising natural biopreservatives in the food industry, particularly in the dairy sector, owing to their ability to enhance microbial safety and extend shelf life without compromising product quality. The LAB demonstrated strong potential for use in cheese production through the secretion of bacteriocins effective against foodborne pathogens such as *L. monocytogenes* [52,53]. Their incorporation in cheese formulations not only improves microbiological stability by inhibiting spoilage and pathogenic microorganisms but also helps maintain sensory attributes. This preservation of organoleptic properties is especially crucial in artisanal and traditional cheeses, where flavor and texture are key factors influencing consumer preference.

5. Conclusions

In this research, LAB were isolated and identified from raw goat milk and assessed for their antimicrobial potential against selected foodborne pathogens. Sequencing of the 16S rRNA gene revealed five isolates belonging to *E. faecium*, *E. durans*, and *L. lactis*. These strains exhibited significant antimicrobial activity against *L. monocytogenes*, a major concern in dairy safety due to its ability to survive refrigeration and cause severe illness. Additionally, some isolates demonstrated inhibitory effects against other important spoilage and pathogenic microorganisms, including *P. aeruginosa*, *B. subtilis*, and *S. aureus*, suggesting a broad-spectrum antimicrobial capacity. Importantly, none of the isolates showed hemolytic or gelatinase activity, nor did they produce histamine, which collectively indicates a favorable safety profile suitable for food applications.

These findings represent an important step toward identifying effective LAB strains for biopreservation. To fully harness their application potential, future research should focus on the purification and comprehensive biochemical characterization of the bacteriocins and other antimicrobial metabolites produced by these strains. Understanding their molecular structure, mechanisms of action, and stability under various food processing and storage conditions will be essential for their effective integration into industrial processes.

Moreover, it is crucial to evaluate the effectiveness of these strains in real food matrices under realistic storage and production conditions. Such trials should assess not only microbial inhibition, but also the impact of LAB on sensory attributes (e.g., flavor, texture, aroma) and product shelf life. Investigating their interactions with the native microbiota and technological parameters will be vital for successful incorporation into commercial cheese production.

In conclusion, the selected LAB strains demonstrate both safety and functionality, supporting their application as natural preservatives in the dairy industry. Their future integration into sustainable food preservation strategies may contribute to cleaner-label products, enhanced food safety, and extended shelf life of both artisanal and industrial fermented cheeses.

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Abbreviations

The following abbreviations are used in this manuscript:

LAB	Lactic Acid Bacteria
BLAST	Basic Local Alignment Search Tool
MRS	De Man, Rogosa and Sharpe
BEA	Bile Esculin Agar
BHI	Brain Heart Infusion
MHA	Mueller-Hinton Agar
TSA	Tryptic Soy Agar

Appendix A

Table A1. Antimicrobial Activity of Lactic Acid Bacteria Isolates Against Indicated Strains using the Agar Spot Test.

Strains	KB1	KB2	KB3	KB4	KB5	KB6	KB7	KB8	KB9	KB10	KB11	KB12	KB13	KB14	KB15	KB16	KB17	KB18
<i>L. monocytogenes</i> CECT 7467	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	+	+	+++	+++	+++	+++	+++
<i>L. monocytogenes</i> CECT 4032	+	+++	++	++	+++	++	+++	+	+	+	+	+	+	++	++	++	++	++
<i>L. monocytogenes</i> CECT 5725	+++	+++	+++	+++	+++	+++	+++	+	+	+	+	+	+	++	+++	+++	++	++
<i>L. monocytogenes</i> CECT 935	+	+++	++	++	++	++	+++	+	+	+	+	+	+	++	++	++	++	++
<i>S. aureus</i> CECT 976	+++	++	+++	+++	++	+++	++	+	+	+	+	+	+	++	++	++	++	++
<i>E. coli</i> CECT 4076	+	+	++	+	+	+	+	+	+	++	+	+	+	++	+	++	++	++
<i>E. coli</i> ATCC 25922	+	+	+	+	+	++	++	*	*	*	*	*	*	++	++	++	++	++
<i>B. subtilis</i> DSMZ 6633	++	+	+	+	+	+	+	*	*	*	*	*	*	++	++	++	++	++
<i>S. enterica</i> CECT 704	++	++	++	++	++	++	++	*	*	*	*	*	*	++	+++	++	++	++
<i>P. aeruginosa</i> CECT 118	+++	+	++	++	+	++	++	*	*	*	*	*	*	+++	++	++	++	++

Sensitivity was expressed as the size of inhibition zones: *: unclear halo, -: No inhibition, + < 15mm, ++ between 15 and 20 mm, +++ > 20mm. DSMZ, German Collection of Microorganisms and Cell Cultures; CECT, Spanish Type Culture Collection; ATCC: American Type Culture Collection.

Table A2. Inhibition Effect of Selected LAB against Foodborne Pathogens using the Agar Well Diffusion Test.

Strains	KB1	KB2	KB3	KB4	KB5	KB6	KB7	KB8	KB9	KB10	KB11	KB12	KB13	KB14	KB15	KB16	KB17	KB18
<i>L. monocytogenes</i> CECT 7467	++	++	++	++	++	++	++	++	++	++	++	++	++	++	++	+	++	++
<i>L. monocytogenes</i> CECT 4032	++	++	++	++	++	++	++	++	++	+	+	+	+	+	++	++	++	+
<i>L. monocytogenes</i> CECT 5725	++	++	++	++	++	++	++	++	++	+	+	+	+	++	++	++	+	+
<i>L. monocytogenes</i> CECT 935	++	++	++	++	++	++	++	++	++	+	+	+	+	+	+	+	+	+
<i>S. aureus</i> CECT 976	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	+	+	+
<i>E. coli</i> CECT 4076	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>E. coli</i> ATCC 25922	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>B. subtilis</i> DSMZ 6633	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	-
<i>S. enterica</i> CECT 704	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
<i>P. aeruginosa</i> CECT 118	++	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Sensitivity was expressed as the size of inhibition zones: (-) no inhibition; (+) <15 mm; (++) between 15 and 20 mm.

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