International Journal of Food Microbiology 419 (2024) 110752

Contents lists available at ScienceDirect

International Journal of Food Microbiology

journal homepage: www.elsevier.com/locate/ijfoodmicro

Selection of starter lactic acid bacteria capable of forming biofilms on wooden vat prototypes for their future application in traditional Sicilian goat's milk cheese making

Fanny Claire Capri [a], Raimondo Gaglio [b,*], Luigi Botta [c], Luca Settanni [b], Rosa Alduina [a,d]

[a] Department of Biological, Chemical and Pharmaceutical Sciences and Technologies (STEBICEF), University of Palermo, Viale delle Scienze Bldg. 16-17, 90128 Palermo, Italy
[b] Department of Agricultural, Food and Forest Sciences (SAAF), University of Palermo, Viale delle Scienze, Bldg. 5, 90128 Palermo, Italy
[c] Department of Engineering, RU INSTM, University of Palermo, Viale delle Scienze, Bldg. 6, 90128 Palermo, Italy
[d] National Biodiversity Future Center (NBFC), Piazza Marina, 61, 90133 Palermo, Italy

ARTICLE INFO

Keywords:
Bacteriocin
Lactic acid bacteria
Microbial biofilm
Raw goat milk
Traditional cheese
Wooden vat surfaces

ABSTRACT

In this study, 327 presumptive lactic acid bacteria (LAB) were isolated from goats' milk acid curds produced at a Sicilian dairy farm with the aim to identify potential starter cultures for traditional cheeses. All isolates were first processed by randomly amplified polymorphic DNA (RAPD)-PCR analysis. This approach identified 63 distinct strains which were evaluated for their acidifying capacity. Only 15 strains specifically stood out for their acidification capacity and were identified through 16S rRNA gene sequencing as *Lactococcus lactis* (11 strains) *Enterococcus faecalis* (three strains), and *Ligilactobacillus animalis* (one strain). Notably, all 15 LAB isolates produced bacteriocin-like inhibitory substances and anti-biofilm compounds, against both planktonic and biofilm forms of *Listeria monocytogenes*, *Salmonella* Enteritidis, *Escherichia coli*, and *Staphylococcus aureus*, albeit at varying levels. Among these 15 LAB, *En. faecalis* RGM25 and *Lc. lactis* RGM55, susceptible to five antibiotics tested, were put in contact with wooden vat prototypes, because all equipment used in traditional cheese production in Sicily are made of wood. Scanning electron microscopy and bacterial plate counts of the wooden vat prototypes showed the development of biofilms at levels of approximately 6.0 log CFU/cm^2. Overall, this study contributes to establishing a custom-made LAB starter cultures with bio-preservatives properties for Sicilian cheese productions.

1. Introduction

The growing awareness and higher expectations of consumers regarding the quality of agri-food products have given rise to a market of items strongly associated with specific regions; examples include traditional cheeses made in distinct geographical areas. Goat milk, known for its excellent digestibility, nutritional richness, and lower allergenic compounds compared to cow's milk, plays a vital role in a healthy diet, particularly for children and the elderly (Yang et al., 2023).

Cheese production technology hinges on two key processes: lactic acid coagulation facilitated by lactic bacteria (LAB) and rennet coagulation catalysed by enzymes found in animal rennet. In the cheese-making process, starter LAB (SLAB) play a crucial role in curd acidification, while non-starter LAB (NSLAB) contribute to maturation (Settanni and Moschetti, 2010). When specific starter cultures are not directly inoculated into the milk, the primary sources of SLAB include the milk's microbiota, equipment, animal rennet, and the processing environment (Beresford et al., 2001; Franciosi et al., 2009). Raw milk and the entire cheese production process can harbour pathogenic bacteria, posing a risk of contaminating the final dairy products. Key factors crucial for ensuring cheese safety encompass milk quality, LAB, pH levels, salt content, control of ripening conditions, and the chemical transformations that occur during cheese maturation. To enhance cheese hygiene, a strategic approach involves utilizing bacteriogenic LAB as starters or co-cultures (Settanni and Moschetti, 2014). Numerous studies have highlighted the production of bacteriocins by SLAB, which serve as effective biopreservatives for cheeses (Hernández et al., 2005; Niederhäusern et al., 2020; O'Sullivan et al., 2006; Trejo-González et al., 2022). Additionally, the by-products resulting from SLAB catabolism not only aid in cheese preservation but also contribute to its flavour, aroma,

* Corresponding author.
E-mail address: raimondo.gaglio@unipa.it (R. Gaglio).

https://doi.org/10.1016/j.ijfoodmicro.2024.110752
Received 22 February 2024; Received in revised form 13 May 2024; Accepted 16 May 2024
Available online 20 May 2024

and texture, thereby playing a pivotal role in defining the unique characteristics of the final product (Guarcello et al., 2016; Monfredini et al., 2012).

Utilizing autochthonous bacteria specifically adapted to the production region, local raw materials, and traditional protocols impart distinctive characteristics to cheeses (Settanni and Moschetti, 2014). In Sicily, where most traditional cheeses are crafted, raw milk from native breeds serves as the primary ingredient. The cheese-making process involves the use of wooden equipment (Busetta et al., 2023a; Busetta et al., 2023b; Gaglio et al., 2016a; Scatassa et al., 2015). Furthermore, the ripening of these cheeses takes place on wooden shelves (Settanni et al., 2021; Wadhawan et al., 2021), aligning with European Regulation (EC) No 2074/2005, which recognizes and preserves the unique features of traditional food products (Commission Regulation, 2005). Wood is commonly used in cheese production in France and Italy, and several studies have confirmed its microbiological safety (Busetta et al., 2023a; Cruciata et al., 2018; Gaglio et al., 2016a; Lortal et al., 2014; Scatassa et al., 2015; Sun and D'Amico, 2023a, 2023b). Investigations into wooden surface biofilms consistently reveal the presence of desired dairy LAB while excluding pathogenic species like *Listeria monocytogenes* and *Salmonella* spp. Traditional Sicilian cheeses, ripened on wooden vat surfaces, benefit from the full spectrum of dairy LAB species essential for both fermentation (Lortal et al., 2009; Scatassa et al., 2015; Settanni et al., 2012) and maturation (Di Grigoli et al., 2015).

LAB exhibit an affinity for wooden surfaces, adhering to both each other and the wood through their self-produced matrix of extracellular polymeric substances (EPS). This collective assembly of microorganisms is known as a biofilm (Vert et al., 2012). Biofilms naturally immobilize cells when microorganisms attach to solid surfaces - whether biotic or abiotic - in submerged environments. The high cell density within the biofilm enables bacteria to withstand various stressors, including pH fluctuations and periods of nutrient lack (Tatsaporn and Kornkanok, 2020).

This study aimed to select custom-made LAB starter cultures from acid curds. After isolation, LAB were characterised based on their technological properties and food safety and, final, applied onto wooden vat prototypes to evaluate their ability to form biofilms necessary in traditional Sicilian cheese making.

2. Materials and methods

2.1. Isolation of LAB from acid curd samples

Curd samples were collected during cheese making at Tenuta Manchi farm, located in Caccamo (Palermo, Italy). Specifically, eight curd samples were obtained from a single producer following a traditional production protocol that excluded the addition of starter LAB in eight production days. The milk samples were obtained from the same *Camosciata delle Alpi* goat breed during various lactation periods (specifically, in July and October 2021, and March and July 2022), and the curds were sampled approximately 24 h after rennet was added. The curds were carefully transferred into sterile plastic bags and transported to the laboratory under refrigeration. Once at the laboratory of Agricultural Microbiology – University of Palermo – the curds were divided into two aliquots from each sample and kept at 30 °C and 44 °C. When the pH dropped to 3.5–4.0, 10 g of each acidified curd was homogenised in a 90 mL sodium citrate solution (2 %, *w/v*) using a Stomacher (Bag-Mixer® 400, Interscience, Saint Nom, France) at the maximum speed. The curd suspensions were serially diluted (at a 1:10 ratio) in Ringer's solution. Cell suspensions were then plated on agar media to cultivate different microbial groups. Total mesophilic microorganisms (TMM) were cultured on plate count agar (PCA) supplemented with 1 g/L skim milk (SM); this medium is commonly used for dairy samples under aerobic incubation at 30 °C for 72 h. Thermophilic and mesophilic LAB cocci were grown on M17 agar. These cultures were incubated anaerobically in hermetically sealed jars at 44 and 30 °C, respectively, for 48 h. Thermophilic and mesophilic LAB rods were cultivated on de Man-Rogosa-Sharpe (MRS) agar, which was acidified to pH 5.4 with lactic acid (5 M). These cultures were also incubated anaerobically at 44 and 30 °C for 48 h. All media and supplements were purchased from Oxoid (Milan, Italy). Plate counts were performed in duplicate. Following these steps, presumptive LAB colonies were characterised using the method outlined by Settanni et al. (2012). Briefly, LAB colonies were purified by successive sub-culturing, and the purity was checked microscopically. The isolates were phenotypically characterised firstly by determining the Gram type after treatment with 3 % (*w/v*) KOH and the production of catalase enzyme by transferring a fresh colony to a glass slide followed by addition of 5 % (*v*/v) H_2O_2. Rods and cocci were then differently evaluated: LAB rods were grouped based on their cell arrangement, growth at 15 and 45 °C and CO_2 production from glucose, while LAB cocci were tested for growth at pH 9.2 and with 6.5 % (w/v) NaCl.

2.2. DNA extraction, genotypic differentiation, and identification of LAB

Cell lysis for DNA extraction was performed using the Instagene Matrix kit (Bio-Rad, Hercules, CA) as described by the manufacturer. Strain differentiation was performed by random amplification of polymorphic DNA-PCR (RAPD-PCR) analysis in a 25-μL reaction mix using single primers M13 (Stenlid et al., 1994), AB111, and AB106 as reported by Gaglio et al. (2017). The polymorphic profiles resulting from PCR were analysed using GelCompare II software version 6.5 (Applied-Maths, Saint-Marten-Latem, Belgium). This software helps to assess the similarity among PCR pattern products. Thus, the isolates exhibiting significant similarity are interpreted as representing the same strain. Genotypic identification of LAB with different RAPD-PCR profiles was performed using 16S rRNA gene amplification and sequencing. DNA was used to amplify the 16S rRNA gene (1500 bp) using the primers F1 (GAGTTTGATCCTGGCTCAG) and R12 (ACGGCTACCTTGTTACGACT) (Coy et al., 2014). The resulting DNA amplicons were verified by electrophoresis on 1 % *w/v* agarose gel, followed by purification using the QIAquick purification kit (Qiagen S.p.a., Milan, Italy). Subsequently, these amplicons were sequenced using the same primers employed for PCR amplification, following the Sanger method at BMR Genomics s.r.l. (Padova, Italy). The sequence outputs were analysed using the alignment tool (BLAST), revealing a percentage of identity with sequences available in the NCBI database of at least 99 %. The sequence dataset was deposited in the GenBank database under Acc. No. PP130098 – PP130112.

2.3. Acidification capacity

The 63 LAB isolates were grown overnight in either M17 or MRS medium and then collected by centrifugation at 5000 ×*g* for 5 min. The resulting cell pellet was washed with Ringer's solution (Sigma-Aldrich, Milan, Italy) and subsequently resuspended in the same solution. To evaluate their acidifying capacity, each strain of LAB was tested using 10 mL of full-fat ultra-high temperature (UHT) goat's milk (Biancoviso, Saluzzo, Italy). The milk was inoculated with 1 % (*v*/v) of the cell suspension, achieving a final concentration of approximately 10^7 CFU/mL. The incubation took place at 30 °C, and pH measurements were recorded at 2-h intervals during the initial 8 h, followed by measurements at 24, 48, and 72 h after inoculation (Settanni et al., 2013).

2.4. Antibiotic resistance determination

The minimum inhibitory concentration (MIC) of five different antibiotics [ampicillin (AMP), vancomycin (VA), tetracycline (TE), erythromycin (E), ceftriaxone (CTX)] (Oxoid) commonly used in veterinary medicine, was determined for 15 LAB. These antibiotics represent various classes and were tested using the broth dilution method on 96-well plates. The MIC values were then compared to the established cut-off values for resistance, as defined in the guidelines for *Lactococcus*

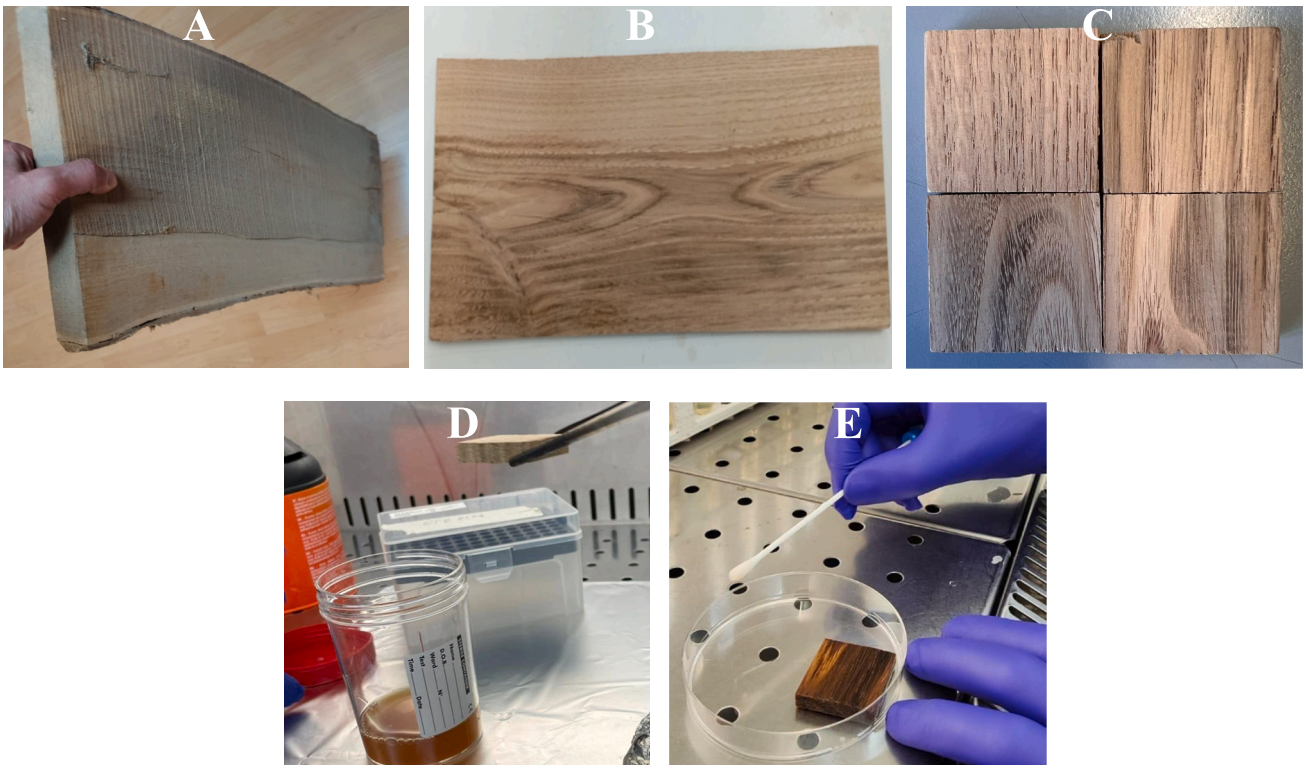

Fig. 1. Schematic preparation of wooden vat prototype and biofilm sampling: A, raw beech wood board; B, planed beech wood board; C, squares of planed beech wood planks measuring 3 × 3 cm; D, insertion of the wooden squares into the bacterial suspension; E, biofilm sampling following 48 h of contact with bacterial suspensions.

spp., *Ligilactobacillus* spp., and *Enterococcus* spp. by the Clinical and Laboratory Standards Institute (CLSI, 2020). Isolates demonstrating resistance to three or more antibiotic classes were classified as multidrug-resistant (MDR). For isolates resistant to tetracycline, a PCR-colony assay was conducted to detect specific antibiotic resistance genes (*tetA*, *tetC*, *tetD* and *tetW*) using DreamTaq DNA Polymerase (Thermo Fisher Scientific, Beverly, MA, USA) following the manufacturer's instructions and the following primers: tetA-F (GCTACATCCTGCTTG CCTTC) and tetA-R (CATAGATCGCCGTGAAGAGG), tetC-F (CTTGA-GAGCCTTCAACCCAG) and tetC-R (ATGGTCGTCATCTACCTGCC), tetD-F (AAACCATTACGGCATTCTGC) and tetD-R (GACCGGATACACCAT CCATC), tetW-F (ACATCATTGATACTCCAGGTCACG) and tetW-R (TTTCACTTTGTGGTTGAACCCCTC). *En. faecalis* ATCC49532 was used as a positive control.

2.5. Production of bacteriocin-like inhibitory substances

The antibacterial activity of 15 LAB was assessed against four food-borne pathogen strains: *L. monocytogenes* ATCC19114, *Salmonella* Enteritidis ATCC13076, *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC33862 (Table 2). The inhibitory activities were evaluated using the well diffusion assay (WDA), following the methodology modified by Corsetti et al. (2008). All experiments were performed in triplicate, and streptomycin served as the positive control. The supernatants showing inhibitory properties were treated with proteinase K (12.5 U/mg), protease B (45 U/mg), and trypsin (10.6 U/mg) diluted to 1 mg/mL in phosphate buffer (pH 7.0) to verify the proteinaceous nature of the active substances. After incubating at 37 °C for 2 h, the remaining activity was measured by a second WDA. All enzymes were purchased from Sigma-Aldrich.

2.6. Crystal-violet biofilm assay

The ability of 15 LAB isolates to form biofilms was assessed as described previously (Capri et al., 2023). Briefly, axenic pre-cultures of selected LAB were inoculated in either MRS or M17 broth media. Bacterial growth occurred over approximately 48 h at 30 °C. Each bacterial isolate was then inoculated (1 % *v*/v, corresponding to ca 1 × 10^7 CFU/mL) into 200 µL of fresh MRS or M17 media. These cultures were aliquoted into 96-well flat-bottom polystyrene plates. The plates were incubated under static conditions at 30 °C for 24 h. After this incubation period, the absorbance values of planktonic cells were measured at 600 nm using a microplate reader (BioTek® - Synergy HT, Santa Clara, CA, USA). The suspension was discarded, and the wells underwent three washes with sterile water to eliminate any trace of loosely attached cells. Bacterial biofilms were stained with 200 µL solution of crystal violet (0.1 % *w/v*) in water, allowing the staining process to occur for 15 min at room temperature. After staining, the wells were once again washed with sterile water to remove any unbound dye. The wells were then dried at 65 °C for 30 min. Finally, crystal violet was dissolved using a 200 µL solution of acetic acid (33 % *v*/v) in water, and the absorbance was measured at 600 nm. The specific biofilm formation (SBF) index was calculated using the formula reported by Capri et al. (2023). *En. faecalis* ATCC49532 was used as a positive control.

2.7. Anti-biofilm activity of LAB supernatant against pathogenic biofilm formation

The overnight cultures of 15 LAB underwent centrifugation at 10,000 ×*g* for 10 min to separate cell pellets and supernatants. The supernatants were then filtered using a 0.45 µm microfilter (Millipore, Billerica, MA, USA). Subsequently, 1 mL of the supernatant was combined with 1 mL of an overnight culture containing *L. monocytogenes* ATCC19114, *S.* Enteritidis ATCC13076, *E. coli* ATCC25922, and *St.*

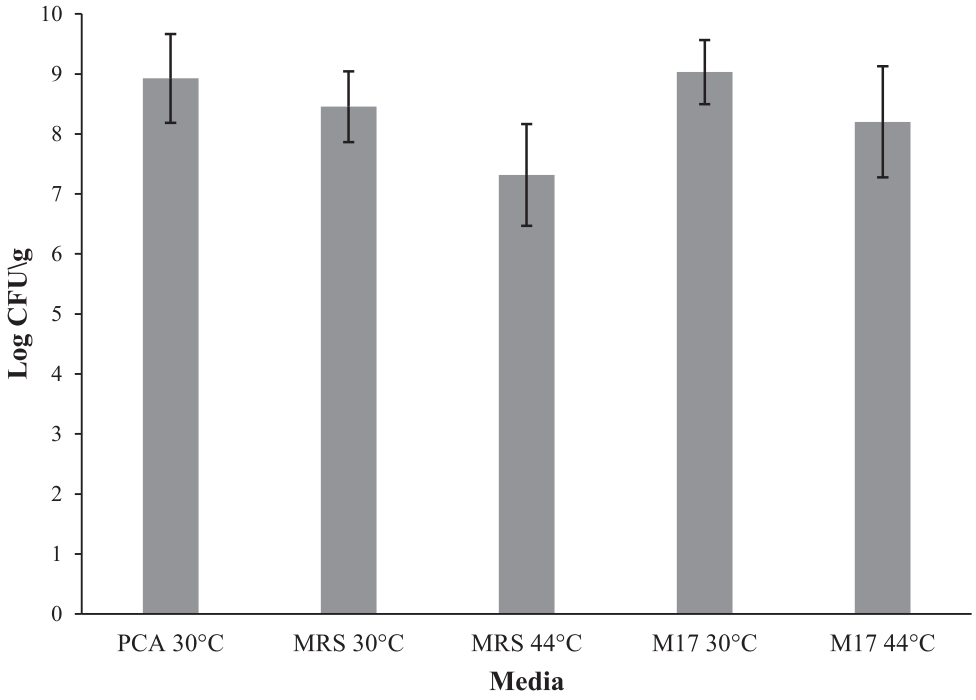

Fig. 2. Microbial load (log CFU/g) of acidified curd samples. Results indicate mean values ± standard deviations. Abbreviations: PCA 30 °C, plate count agar added with skim milk incubated at 30 °C detection of total mesophilic counts; MRS, de Man-Rogosa-Sharpe agar for detection of mesophilic (30 °C) and thermophilic (44 °C) rod LAB; M17, medium 17 agar for detection of mesophilic (30 °C) and thermophilic (44 °C) coccus LAB.

aureus ATCC33862 (10^7–10^8 CFU/mL). These cultures, along with the LAB supernatants, were incubated at 37 °C for 24 h. After incubation, the resulting biofilms were stained with crystal violet (as described above). The optical density at 600 nm was measured using a microplate reader (BioTek® - Synergy HT). Finally, the biofilm-forming ability under each condition was compared based on the intensity of the crystal violet staining. A *t*-test was conducted to assess statistical significance (*p-value* < 0.05).

2.8. Biofilm production on wooden surfaces

Two LAB (*Lc. lactis* RGM25 and *En. faecalis* RGM55) were pre-cultured due to their excellent technological properties and safe characteristics for food applications. The pre-cultures were established by inoculating a single colony into M17 broth and allowing bacterial growth for approximately 48 h at 30 °C. Subsequently, each bacterial isolate and a co-culture of both strains were inoculated (1 % *v*/v, corresponding to approximately 1×10^7 CFU/mL) into 30 mL of fresh M17 medium for another 48 h at the same temperature. Wooden vat prototypes (measuring 3 × 3 cm), representing tanks used in dairy production, were placed in contact with the bacterial cultures for 48 h at 30 °C (Fig. 1). Wooden vat prototypes were prepared from a chestnut board; chestnut wood is historically used to prepare the dairy wooden equipment for cheese making in western Sicily (Di Grigoli et al., 2015; Gaglio et al., 2016a; Scatassa et al., 2015; Settanni et al., 2012). Thus, the wooden squares prepared in this study can be considered as adequate mimetic assay for real wooden vats. The resulting biofilm on the wood surface was collected using a sterile swab and then diluted in Ringer's solution. Additionally, the wood was exposed to 20 mL of commercial UHT goat's milk (Biancoviso) for 15 min to assess any potential release during fermentation. Simultaneously, negative controls were analysed, consisting of the growth medium with wood and milk but without bacterial inoculation. To quantify the bacterial load, cell suspensions from wood surface samples and milk samples underwent decimal serial dilutions in Ringer's solution and were incubated on plate counting agar (PCA) supplemented with 1 g/L skim milk and M17 agar for 48 h at 30 °C.

The plate count data underwent statistical analysis using one-way variance analysis (ANOVA). The software employed for this purpose was XLSTAT, version 2020.3.1 for Microsoft Excel (Addinsoft, New York, NY, USA). The growth medium served as the variable included in the model. To ascertain differences between means, Tukey's test was applied with a significance level of $p < 0.05$.

2.9. Scanning electron microscopy

The formation of microbial biofilms on the wooden vat prototype surfaces was examined using scanning electron microscopy (SEM) (FEI Quanta 200F; FEI, Holland). Specifically, rectangular wood splinters (measuring 50 × 35 mm and 1–2 mm thickness) were aseptically sampled both before and after activation. To prepare the samples, they underwent dehydration following the method outlined by Mallia et al. (2005) and subsequent drying as described by Lortal et al. (2009). These wooden splinters were then mounted with the side that had been in contact with the bacterial suspension facing upward on an aluminium holder. To enhance visualization, all specimens were sputter coated with gold (20 mÅ; 300 s) (Edwards S150A sputter coater) and observed under SEM.

3. Results and discussion

3.1. Microbiological analysis

In this investigation, the LAB community of raw goat milk curds was studied and subjected to acidification at varying temperatures. Based on phenotypic appearance, approximately four colonies per morphology were isolated from each medium used for LAB counts. These media included PCA at 30 °C, MRS, and M17 at 30 °C and 44 °C as depicted in Fig. 2. A total of 327 colonies were isolated and propagated in broth media corresponding to those used for plate counts. After Gram and catalase testing, all isolates were still considered presumptive LAB cultures, characterised by being Gram-positive and catalase-negative. The

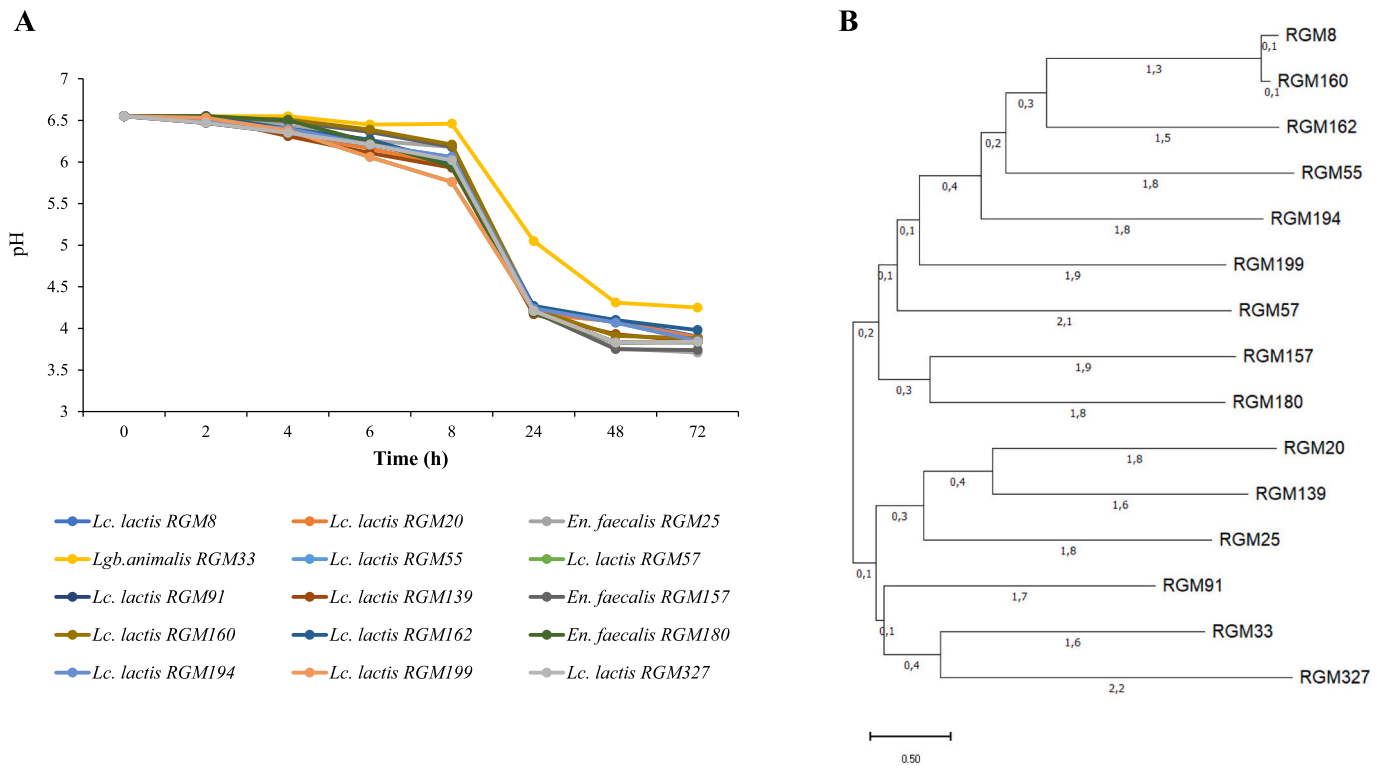

Fig. 3. Acidification kinetics (A) and phylogenetic tree based on 16S rRNA gene (B) of the most performing LAB. Abbreviations: *En.*, *Enterococcus*; *Lc.*, *Lactococcus*; *Lgb.*, *Ligilactobacillus*.

Table 1
Antibiotic resistance profile of LAB.

Species	Strain	Antibiotics				
		Ampicillin	Vancomycin	Tetracycline	Erythromycin	Ceftriaxone
Lc. lactis	RGM8	S	S	R	S	I
Lc. lactis	RGM20	I	S	S	S	S
En. faecalis	RGM25	S	S	S	S	S
Lgb. animalis	RGM33	I	S	n.a.	S	n.a.
Lc. lactis	RGM55	S	S	S	S	S
Lc. lactis	RGM57	S	S	S	S	I
Lc. lactis	RGM91	S	S	I	S	S
Lc. lactis	RGM139	I	S	S	I	I
En. faecalis	RGM157	S	S	R	S	S
Lc. lactis	RGM160	I	S	I	S	I
Lc. lactis	RGM162	I	S	I	S	I
En. faecalis	RGM180	S	S	R	S	S
Lc. lactis	RGM194	I	S	R	S	S
Lc. lactis	RGM199	S	S	S	S	S
Lc. lactis	RGM327	S	S	I	S	S

Abbreviations: *En.*, *Enterococcus*; *Lc.*, *Lactococcus*; *Lgb.*, *Ligilactobacillus*; R., resistant; I., intermediate resistant; S., susceptible; n.a., not analysed.

acidified curds exhibited a cell density of approximately 9 log CFU/g on M17 agar (30 °C), primarily comprising mesophilic cocci LAB. These levels were comparable to those of TMM. Conversely, the counts on MRS agar at 44 °C showed a decrease of at least 2 log cycles, indicating a decline in thermophilic LAB rods. In our study, an increase in mesophilic lactic strains was observed, which could influence the sensory profile of dairy products and their final yield (Ayivi et al., 2020; Coelho et al., 2022). Furthermore, the decrease in thermophilic strains is probably due to the autolytic capacities of these bacteria. *Streptococcus thermophilus* strains are capable of growing normally and undergoing extensive lysis at the end of growth, under appropriate conditions (Husson-Kao et al., 1999, 2000). The autolytic capacity favours the activity of the intracellular enzymes involved in the formation of aromatic compounds, allowing their release into the curd, and influencing the sensory properties (Husson-Kao et al., 2000). The autochthonous microbial community in goat's milk may likely originate primarily from utensils, environmental conditions, and preservation techniques. These factors may have contributed to the dominance of lactococci (Perin and Nero, 2014). To further investigate, RAPD analysis was conducted on all isolates using three single primers. The results revealed a total of 63 distinct strains among the goat's milk curd isolates (Fig. S1).

3.2. Technological screening

Before genetic identification, the presumptive 63 LAB isolates underwent assessment for their acidifying capacity. The acidification kinetics (Fig. 3-A) showed that 15 different strains exhibited the fastest decrease in the milk pH. These strains achieved pH values ranging from

Table 2
Bacteriocin-like inhibitory activity of LAB[a].

Species	Strain	Indicator strains[b]			
		ATCC19114	ATCC13076	ATCC25922	ATCC33862
Lc. lactis	RGM8	1.65 ± 0.21	2.05 ± 0.21	–	–
Lc. lactis	RGM20	1.40 ± 0.28	1.80 ± 0.00	1.95 ± 0.21	1.35 ± 0.07
En. faecalis	RGM25	1.45 ± 0.07	1.80 ± 0.14	2.00 ± 0.00	1.75 ± 0.07
Lgb. animalis	RGM33	1.85 ± 0.21	1.85 ± 0.07	1.90 ± 0.14	1.70 ± 0.14
Lc. lactis	RGM55	2.05 ± 0.21	1.90 ± 0.28	1.45 ± 0.07	1.65 ± 0.07
Lc. lactis	RGM57	1.60 ± 0.28	1.50 ± 0.28	–	–
Lc. lactis	RGM91	2.05 ± 0.07	2.15 ± 0.07	–	1.60 ± 0.00
Lc. lactis	RGM139	–	1.65 ± 0.07	1.65 ± 0.07	–
En. faecalis	RGM157	2.15 ± 0.07	2.15 ± 0.07	–	1.75 ± 0.35
Lc. lactis	RGM160	2.35 ± 0.21	2.00 ± 0.00	–	1.55 ± 0.35
Lc. lactis	RGM162	1.85 ± 0.21	1.60 ± 0.00	–	–
En. faecalis	RGM180	2.40 ± 0.14	1.85 ± 0.21	–	–
Lc. lactis	RGM194	1.40 ± 0.14	1.85 ± 0.21	1.75 ± 0.07	1.50 ± 0.14
Lc. lactis	RGM199	2.10 ± 0.00	2.15 ± 0.07	–	1.50 ± 0.14
Lc. lactis	RGM327	2.25 ± 0.21	2.45 ± 0.07	–	1.60 ± 0.14

Results indicate mean ± standard deviations. Symbols: – no inhibition found.
[a] Width of the inhibition zone (millimeters).
[b] Bacterial species: *Listeria monocytogenes* ATCC19114; *Salmonella* Enteritidis ATCC13076; *Escherichia coli* ATCC25922; *Staphylococcus aureus* ATCC33862.

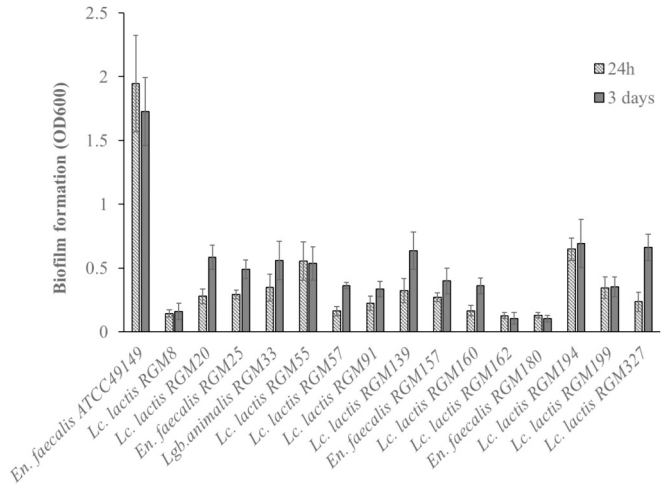

Fig. 4. Biofilm production by LAB after 24 h and 3 d. Results indicate mean values ± standard deviations. *Enterococcus faecalis* ATCC49149 was used as a positive control.

3.76 to 4.51 after 24 h of fermentation. Notably, the bacterial strain RGM199 emerged as the most efficient acidifier, with milk inoculated by this strain reaching a pH of 5.76 within just 8 h. Except for RGM33, all other strains induced a pH drop below 5.00 after 24 h and below 4.5 after 48 h. The release of organic acids, mainly lactic and acetic acids, by LAB, plays a crucial role in lowering the pH, reducing the risk of pathogen contamination, and creating a more favourable environment for the resident microbiota (Hossain et al., 2017). An optimal starter LAB is characterised by rapid acidification, contributing to the texture, flavour, and preservation of dairy products (Ayivi et al., 2020; Coelho et al., 2022; Mayo et al., 2021).

3.3. Genetic identification

All 15 isolates were genetically identified as *Lc. lactis* (11 strains), *Enterococcus faecalis* (three strains), and *Ligilactobacillus animalis* (one strain) (Fig. 3-B). While the presence of *En. faecalis* in dairy products is often linked to fecal contamination, it is worth noting that, according to Commission Regulation (EC) No 1441/2007, enterococci in food do not necessarily indicate such contamination (Commission Regulation, 2007). Enterococci contribute to enhancing organoleptic characteristics and produce bacteriocins, which aid in extending product shelf life. Certain strains of enterococci are also utilized as components in cheese adjunct cultures or as probiotics (Cruciata et al., 2014; Gaglio et al., 2016b; Scatassa et al., 2015). Additionally, *Lactococcus* spp. and *Ligilactobacillus* spp. are commonly found in goat milk, as reported in several studies (De Almeida Júnior et al., 2015; Makete et al., 2017; Picon et al., 2016). Their prevalence over other bacterial strains may be influenced by factors such as goat breeds, environment, and husbandry practices.

3.4. Antibiotic susceptibility of LAB strains

Several studies (Frétin et al., 2018; Obioha et al., 2023; Stefańska et al., 2021; Wang et al., 2019) have demonstrated that LAB strains exhibit antibiotic resistance to different classes of antibiotics, influenced by factors such as the dairy product variety or LAB species. Consequently, the European Food Safety Authority (EFSA) strongly recommends screening starter cultures before commercialization (EFSA, 2018). In our study, the MIC values for all tested antibiotics (Table 1) remained within acceptable limits, as defined by the CLSI guidelines, across all 15 LAB isolates. Notably, no resistance was observed for the five antibiotic classes, except in the case of four strains: specifically, two *Lc. lactis* strains (RGM8 and RGM199) and two *En. faecalis* strains (RGM157 and RGM180) displayed resistance to tetracycline, although the specific resistance genes (*tetA*, *tetC*, *tetD* and *tetW*) were absent in any of the strains.

Antibiotic resistance has been observed in strains of different LAB species isolated from raw milk and artisanal goat cheese in various studies (Budiati et al., 2022; Da Silva et al., 2019; Herreros et al., 2005; Perin and Nero, 2014; Yang et al., 2023). Among these, tetracycline resistance was the most prevalent. These bacteria serve as a potential reservoir of antimicrobial resistance genes (ARGs) that can be horizontally transferred to other commensal bacteria, as well as pathogenic and opportunistic species (Gargano et al., 2021; Sucato et al., 2021; Vitale et al., 2019). Interestingly, LAB may inherently possess resistance to tetracycline (Chang et al., 2011; Lüdin et al., 2018; Rojo-Bezares et al., 2006) due to its absorption and accumulation within their cells (Stefańska et al., 2021).

3.5. Antibacterial activity and biofilm production

To assess the competitive advantages of the 15 LAB, the test was conducted to evaluate their antibacterial compound production against four foodborne pathogen indicators (Table 2). All tested strains exhibited antibacterial activities against at least one indicator pathogen strain.

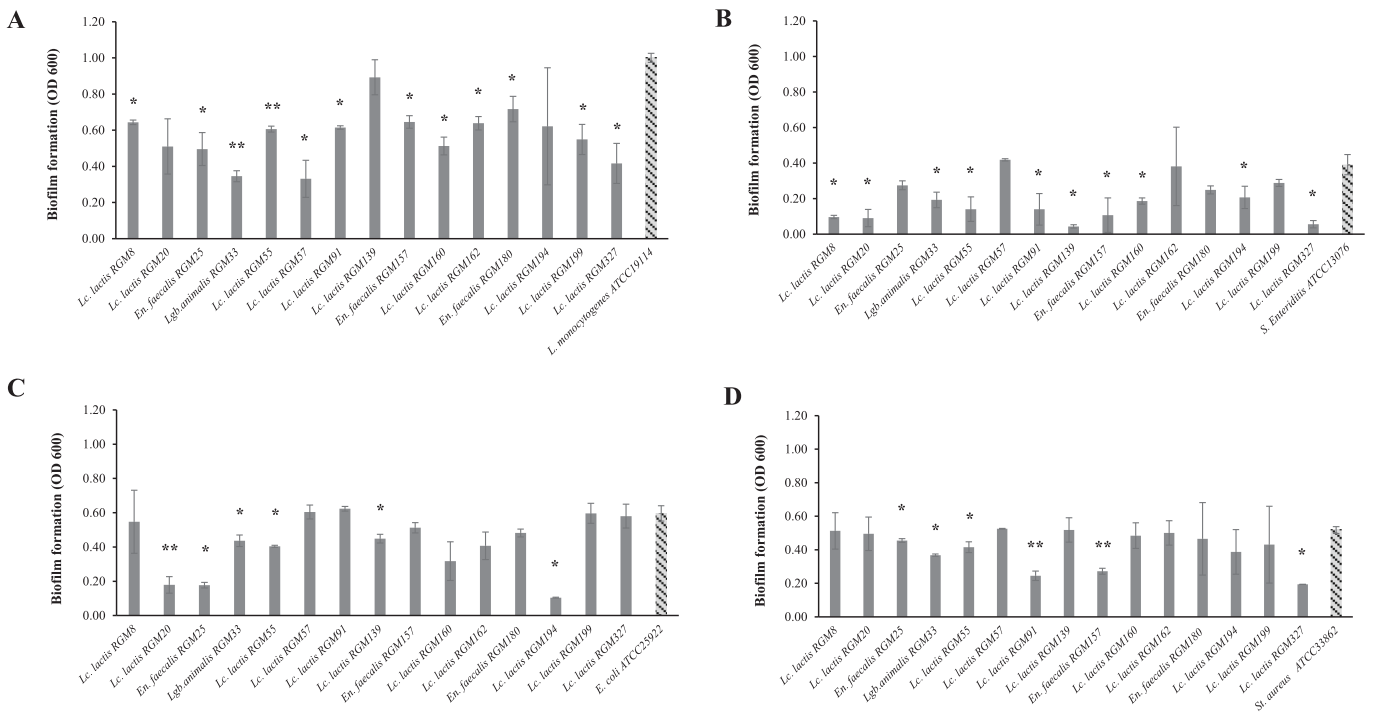

Fig. 5. Anti-biofilm activities of the isolated LAB supernatant against biofilm formations of four foodborne pathogen strains. *Listeria monocytogenes* ATCC19114 (A), *Salmonella* Enteritidis ATCC13076 (B), *Escherichia coli* ATCC 25922 (C), and *Staphylococcus aureus* ATCC 33862 (D). The t-test was used to compare the significant difference in the growth based on OD_{600} (*$p < 0.05$, **$p < 0.001$). Abbreviations: OD, optical density; *En.*, *Enterococcus*; *Lc.*, *Lactococcus*; *Lgb.*, *Ligilactobacillus*.

Table 3
Microbial loads in biofilms of wooden prototypes and goat milk after contact with activated wooden surfaces.

Sample	Bacterial counts	
	PCA 30 °C	M17 30 °C
Wooden:		
RGM25	6.24 ± 0.06 ^{A}	6.27 ± 0.07 ^{A}
RGM55	5.54 ± 0.14 ^{B}	5.62 ± 0.01 ^{B}
RGM25 + RGM55	5.69 ± 0.06 ^{B}	5.76 ± 0.08 ^{B}
p-Value	0.001	0.001
Milk after contact:		
RGM25	6.06 ± 0.03 ^{B}	5.48 ± 0.03 ^{A}
RGM55	6.35 ± 0.07 ^{A}	5.29 ± 0.04 ^{B}
RGM25 + RGM55	6.27 ± 0.01 ^{A}	5.43 ± 0.05 ^{A}
p-Value	0.001	0.005

Units are log CFU/cm^2 for vat surfaces and log CFU/mL for milk samples. Results indicate mean values ± standard deviations. Data within a column followed by the same letter are not significantly different according to Tukey's test.

Consequently, all 15 LAB tested were classified as bacteriogenic. All LAB produced hypothetical bacteriocins specifically targeting *S.* Enteritidis. Among the LAB strains, five (RGM20, RGM25, RGM33, RGM55, and RGM194) showed antibacterial activity against Gram-positive and Gram-negative indicator strains. *Lc. lactis* RGM139 exhibited inhibitory activity primarily against Gram-negative indicator strains (*S.* Enteritidis and *E. coli*) (Table 2). Notably, the bacterial strains RGM20, RGM25 and RGM33 demonstrated the highest inhibition, considering both the number of indicator strains affected and the width of the inhibition areas.

Moreover, all 15 LAB were tested for biofilm formation, since biofilms act as a barrier against the growth of spoilage and pathogenic microorganisms. Biofilm forming capacity of nine LAB strains (RGM20, RGM25, RGM33, RGM57, RGM91, RGM139, RGM157, RGM160, and RGM327) during this period, was higher than the strains RGM55, RGM162, RGM180, RGM194, and RGM199 (Fig. 4). Moreover, the LAB strains RGM20, RGM33, RGM55, RGM139, RGM194, and RGM327 exhibited greater biofilm production compared to the other tested strains.

Bacteriogenic LAB strains play a critical role in ensuring the microbiological safety of raw milk cheeses (Psoni et al., 2007; Schirru et al., 2012). Our findings underscore that strains isolated from acid curd inhibit pathogen proliferation by producing antimicrobial metabolites with a broad range, including organic acids, diacetyl, hydrogen peroxide, ethanol, bacteriocin and bactericidal proteins (Vieco-Saiz et al., 2019). Notably, bacteriocins employ mechanisms such as inhibition of cell wall synthesis or formation of pores in the cell membrane (Miao et al., 2016; Yi et al., 2020). These actions block ATP production and arrest cellular metabolism, which explains the ability of our tested hypothetical-bacteriogenic LAB strains to inhibit the growth of the two Gram-negative pathogens, *S.* Enteritidis and *E. coli*.

3.6. Anti-biofilm activity of LAB against foodborne pathogens

Supernatants of all 15 LAB were tested against the biofilm formation of four foodborne pathogen indicators. From nine different *Lc. lactis* strains (RGM8, RGM55, RGM57, RGM91, RGM160, RGM162, RGM194, RGM199, and RGM327), three *En. faecalis* strains (RGM25, RGM157, and RGM180), and *Lgb. animalis* strain RGM33 showed significant effects on the biofilm formation of *L. monocytogenes* (Fig. 5-A). Additionally, *S.* Enteritidis biofilm development (Fig. 5-B) was significantly influenced by supernatants from eight *Lc. lactis* strains (RGM8, RGM20, RGM55, RGM91, RGM139, RGM160, RGM199, and RGM327), *En. faecalis* strain RGM157 and *Lgb. animalis* strain RGM33 at varying levels. *E. coli* biofilm formation (Fig. 5-C) was affected by supernatants from four *Lc. lactis* strains (RGM20, RGM55, RGM139, and RGM194), *En. faecalis* strains RGM25, and *Lgb. animalis* strain RGM33. In Fig. 5-D, the graph illustrates those supernatants from three *Lc. lactis* strains (RGM55, RGM91, and RGM327), two *En. faecalis* strains (RGM25 and RGM157), and *Lgb. animalis* strain RGM33 led to a reduction in *St. aureus* biofilm production.

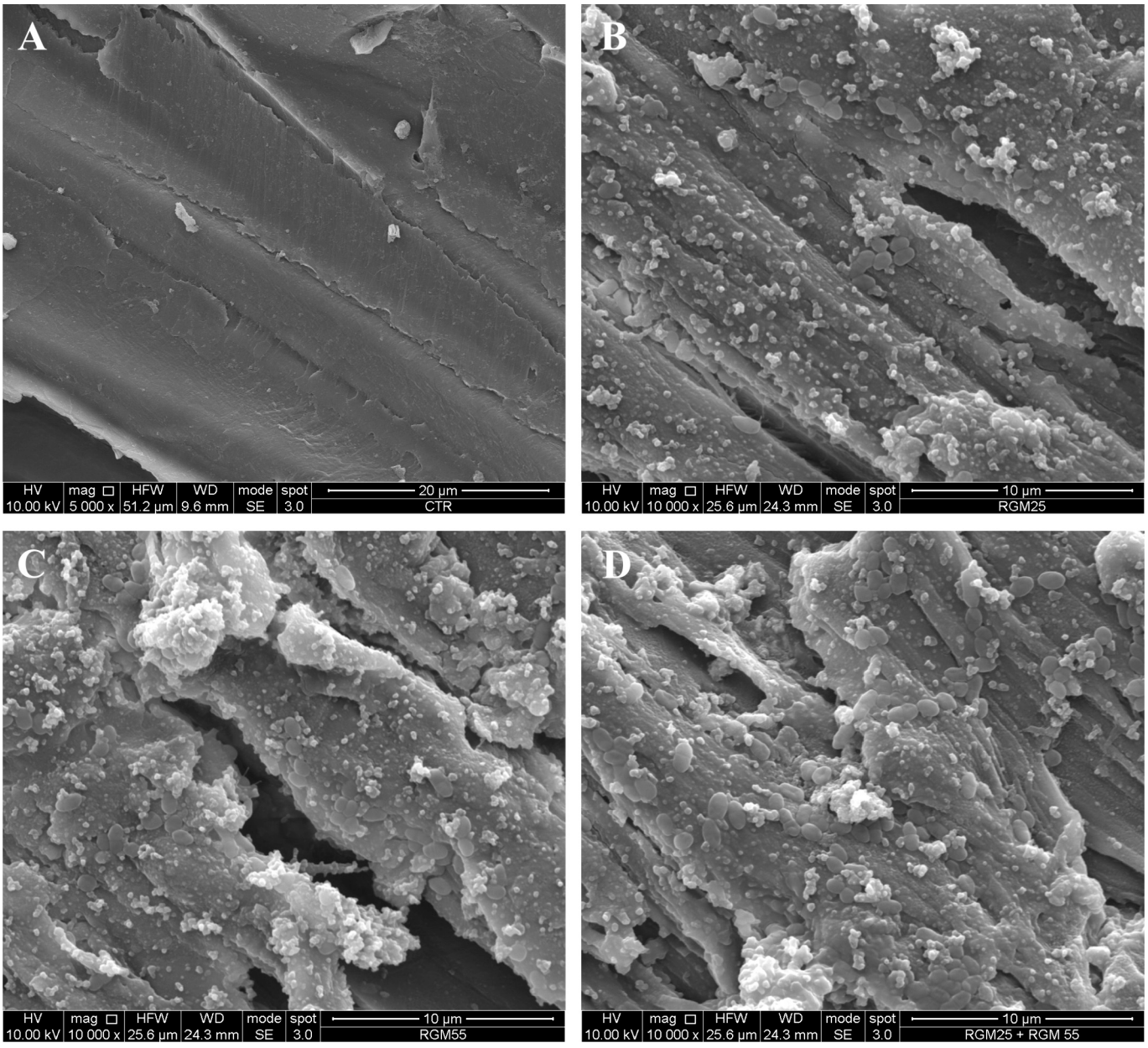

Fig. 6. Scanning electron microscopy observations of wooden splinters. (A) Virgin wood. (B) Wooden prototype activated with the *Enterococcus faecalis* RGM25. (C) Wooden prototype activated with the *Lactococcus lactis* RGM55. (D) Wooden prototype activated with the *Enterococcus faecalis* and *Lactococcus lactis* RGM25 + RGM55 in multi strain combination.

Most of the supernatants from the isolated LAB strains (five strains, accounting for 33.3 %) exhibited activity against two different pathogenic strains. Specifically: the supernatant from *Lc. lactis* strains (RGM20, RGM139, and RGM194) had a significant effect solely on Gram-negative bacterial biofilm formation (Fig. 5-B and C). In contrast, the other supernatants demonstrated broad-spectrum effects across biofilms of both Gram-positive and Gram-negative foodborne pathogens. Notably, *Lactococcus lactis* strain RGM55 and *Ligilactobacillus animalis* strain RGM33 exhibited a similar level of inhibition against biofilm formation for all the tested pathogens.

The complex and compact biofilm matrix produced by pathogens, comprising multiple layers of cells and EPS, hinders the penetration of disinfectants. Thus, eliminating biofilm cells becomes challenging (Al Atya et al., 2016; Sudagidan and Yemenï CïOğlu, 2012). In our study, most of the tested LAB strains effectively reduced biofilms formed by *L. monocytogenes*, *S.* Enteritidis, *E. coli*, and *St. aureus*. This reduction was attributed to the production of antimicrobial metabolites and organic acid. Additionally, previous research has highlighted the bacteriocin-producing abilities of *Lc. lactis* and *En. faecalis* (Ben Braïek et al., 2019; Luo et al., 2021; Raheel et al., 2023; Rocha et al., 2019). These bacteriocins can either inhibit biofilm formation by reducing the population of planktonic cells and bacterial attachment on surfaces or eradicate existing biofilms by inactivating cells within them, thus countering various foodborne pathogens.

Therefore, the combination of different bacteriogenic starters is crucial, especially for raw milk-based dairy products. These co-cultures serve as preservatives, effectively reducing the potential emergence of resistant bacterial communities. Simultaneously, they enhance food safety, quality, and shelf life without compromising the taste and sensory attributes of the products or posing any harm to consumers.

3.7. Activation of wooden vat prototypes

The biofilm formed by the two strains *En. faecalis* RGM25 and *Lc. lactis* RGM55 offers several advantages for industrial applications. These benefits stem from their technological properties, including microbiological safety due to the absence of antimicrobial resistance. Additionally, these strains produce bacteriocin-like compounds that inhibit growth and biofilm formation in tested pathogenic strains. LAB-produced biofilms can serve as a protective shield against pathogens and their associated biofilms. Furthermore, the ability to form biofilms allows LAB to withstand challenging environmental conditions, enhance biomass, and improve the quality of fermented foods.

This study highlights the role of co-cultures in biofilm formation, irrespective of whether the strains exhibit a symbiotic association. *En. faecalis* RGM25 and *Lc. lactis* RGM55 were cultured individually, as well as in a co-culture on wooden vat prototypes to assess biofilm formation and to examine the release of strains from the wood into milk during the preparation steps of dairy products.

In the wooden vat prototypes activated with RGM25, RGM55, and RGM25 + RGM55, levels similar to those commonly found in biofilms that develop in wooden vats used for cheese production were registered (Table 3). LAB densities in milk are consistently affected by biofilms on wooden surfaces, particularly when the initial levels are below 6 log CFU/mL (Didienne et al., 2012; Scatassa et al., 2015). Interestingly, the study by Lortal et al. (2009) demonstrated that these biofilms, which form on wooden vats used in cheese-making, serve as efficient delivery systems for dairy LAB.

The scanning electron microscopy analysis results, obtained from wood splinters collected after 48 h of exposure to different inoculums, are shown in Fig. 6. The wood splinter that was in contact with only M17 medium for 48 h (Fig. 6A) showed no microbial attachment. However, the wood splinter exposed to *En. faecalis* RGM25 (Fig. 6B), *Lc. lactis* RGM55 (Fig. 6C), and the RGM25 + RGM55 co-culture (Fig. 6D) displayed bacterial cocci attachment. These cocci generated a visible exopolysaccharide (EPS) matrix, which is characteristic of biofilm structures on wooden surfaces. The presence of EPS and bacterial cocci corroborated the results obtained from the bacterial count. SEM inspection revealed the absence of bacterial cells on the surfaces of virgin wood, while bacterial aggregates were evident within the EPS matrices. Notably, previous studies have demonstrated that LAB can indeed form biofilms in wooden equipment (Cruciata et al., 2018; Gaglio et al., 2016a; Gaglio et al., 2019; Gaglio et al., 2022; Sun and D'Amico, 2023a, b).

4. Conclusions

This study provided, for the first time, an in-depth characterization and selection of LAB from acidified curds obtained from raw goat's milk. The primary objective was to identify acidifying LAB strains capable of forming biofilms on wooden surfaces during traditional Sicilian goat's milk cheese production. While cow's and sheep's milk cheese production systems have been extensively studied, goat's milk cheese production remains an area that requires further investigation.

Our research findings revealed that only two strains (*En. faecalis* RGM25 and *Lc. lactis* RGM55) were able to form biofilms on wooden vat prototypes and release cells into milk. For this reason, the application of these two LAB strains in co-cultures might enhance production processes, improve cheese quality, and enhance food safety in producing Sicilian raw goat's milk cheeses. Further studies will be carried out to perform a microbial activation of wooden vats with the strains selected in the present study, in order to stabilize traditional Sicilian raw goat's milk cheese production.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2024.110752.

CRediT authorship contribution statement

Fanny Claire Capri: Writing – original draft, Software, Investigation, Formal analysis, Data curation. **Raimondo Gaglio:** Writing – review & editing, Validation, Methodology, Conceptualization. **Luigi Botta:** Formal analysis, Data curation. **Luca Settanni:** Writing – review & editing, Supervision, Conceptualization. **Rosa Alduina:** Writing – review & editing, Validation, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that there is no conflict of interest for this research.

Data availability

Data will be made available on request.

Acknowledgments

This research has been financially supported by the European Commission – NextGenerationEU, Project SUS-MIRRI.IT "Strengthening the MIRRI Italian Research Infrastructure for Sustainable Bioscience and Bioeconomy", code n. IR0000005PO. This research was supported by European Commission – NextGenerationEU, Piano Nazionale Resistenza e Resilienza (PNRR) - Missione 4 Componente 2 Investimento 1.4 – Avviso N. 3138 del 16 dicembre 2021 rettificato con D.D. n.3175 del 18 dicembre 2021 del Ministero dell'Università e della Ricerca – CN5 "National Biodiversity Future Center" – NBFC – code n. CN00000033. The authors wish also to thank MUR, Piano Stralcio "Ricerca e innovazione 2015–2017" – Asse "Capitale Umano", Fondo per lo Sviluppo e la Coesione (FSC) D.D. MIUR prot. n.376 del 315 22/12/2020 DOT1720429 CUP: B73D20005170001 for funding the FCC fellowship and Tenuta Manchi and Salvatore Muscia for their collaboration and supply of raw materials useful for this study.

References

Al Atya, A.K., Belguesmia, Y., Chataigne, G., Ravallec, R., Vachée, A., Szunerits, S., Boukherroub, R., Drider, D., 2016. Anti-MRSA activities of enterocins DD28 and DD93 and evidence on their role in the inhibition of biofilm formation. Front. Microbiol. 7, 817. https://doi.org/10.3389/fmicb.2016.00817.

Ayivi, R.D., Gyawali, R., Krastanov, A., Aljaloud, S.O., Worku, M., Tahergorabi, R., Silva, R.C.D., Ibrahim, S.A., 2020. Lactic acid bacteria: food safety and human health applications. Dairy 1 (3), 202–232. https://doi.org/10.3390/dairy1030015.

Ben Braïek, O., Merghni, A., Smaoui, S., Mastouri, M., 2019. *Enterococcus lactis* Q1 and 4CP3 strains from raw shrimps: potential of antioxidant capacity and anti-biofilm activity against methicillin-resistant *Staphylococcus aureus* strains. LWT-Food Sci. Technol. 102, 15–21. https://doi.org/10.1016/j.lwt.2018.11.095.

Beresford, T.P., Fitzsimons, N.A., Brennan, N.L., Cogan, T.M., 2001. Recent advances in cheese microbiology. Int. Dairy J. 11 (4–7), 259–274. https://doi.org/10.1016/S0958-6946(01)00056-5.

Budiati, T., Suryaningsih, W., Yudiastuti, S.O.N., 2022. The antibiotic resistance of lactic acid bacteria isolated from kefir made from Etawah goat milk. IOP Conf. Ser. Earth Environ. Sci. 980 (1), 012050 https://doi.org/10.1088/1755-1315/980/1/012050.

Busetta, G., Gaglio, R., Mangione, G., Garofalo, G., Franciosi, E., Gannuscio, R., Caccamo, M., Todaro, M., Di Gerlando, R., Settanni, L., Licitra, G., 2023a. Effect of commission implementing regulation (EU) 2020/1319 on the bacterial composition of PDO Provola dei Nebrodi cheese. Int. J. Food Microbiol. 394, 110188 https://doi.org/10.1016/j.ijfoodmicro.2023.110188.

Busetta, G., Garofalo, G., Barbera, M., Di Trana, A., Claps, S., Lovallo, C., Franciosi, E., Gaglio, R., Settanni, L., 2023b. Metagenomic, microbiological, chemical and sensory profiling of Caciocavallo Podolico Lucano cheese. Food Res. Int. 169, 112926 https://doi.org/10.1016/j.foodres.2023.112926.

Capri, F.C., Di Leto, Y., Presentato, A., Mancuso, I., Scatassa, M.L., Alduina, R., 2023. Characterization of *Staphylococcus* species isolates from sheep milk with subclinical mastitis: antibiotic resistance, enterotoxins, and biofilm production. Foodborne Pathog. Dis. https://doi.org/10.1089/fpd.2023.0003 fpd.2023.0003.

Chang, Y.C., Tsai, C.Y., Lin, C.F., Wang, Y.C., Wang, I.K., Chung, T.-C., 2011. Characterization of tetracycline resistance lactobacilli isolated from swine intestines at western area of Taiwan. Anaerobe 17 (5), 239–245. https://doi.org/10.1016/j.anaerobe.2011.08.001.

Clinical and Laboratory Standards Institute, 2020. Performance Standards for Antimicrobial Susceptibility Testing, Twenty-Fifth Informational Supplement in M100-S30. Clinical and Laboratory Standards Institute, Wayne, PA.

Coelho, M.C., Malcata, F.X., Silva, C.C.G., 2022. Lactic acid bacteria in raw-milk cheeses: from starter cultures to probiotic functions. Foods 11 (15), 2276. https://doi.org/10.3390/foods11152276.

Commission Regulation, 2005. No 2074/2005 of 5 December 2005 Laying Down Implementing Measures for Certain Products under Regulation (EC) No 853/2004 of the European Parliament and of the Council and for the Organisation of Official Controls under Regulation (EC) No 854/2004 of the European Parliament and of the Council and Regulation (EC) No 882/2004 of the European Parliament and of the Council, derogating from Regulation (EC) No 852/2004 of the European Parliament and of the Council and amending Regulations (EC) No 853/2004 and (EC) No 854/2004. Off. J. Eur. Union. 338, 27–59.

Commission Regulation, 2007. No 1441/2007 of 5 December 2007 amending Regulation (EC) No 2073/2005 of 15 November 2005 on microbiological criteria for foodstuffs. Off. J. Eur. Union. 338, 1–29.

Corsetti, A., Settanni, L., Braga, T.M., Lopes, M.D.F.S., Suzzi, G., 2008. An investigation of the bacteriocinogenic potential of lactic acid bacteria associated with wheat (*Triticum durum*) kernels and non-conventional flours. LWT-Food Sci. Technol. 41 (7), 1173–1182. https://doi.org/10.1016/j.lwt.2007.07.022.

Coy, M.R., Hoffmann, M., Kingdom Gibbard, H.N., Kuhns, E.H., Pelz-Stelinski, K.S., Stelinski, L.L., 2014. Nested-quantitative PCR approach with improved sensitivity for the detection of low titer levels of *Candidatus* Liberibacter asiaticus in the Asian citrus psyllid, *Diaphorina citri* Kuwayama. J. Microbiol. Methods 102, 15–22. https://doi.org/10.1016/j.mimet.2014.04.007.

Cruciata, M., Sannino, C., Ercolini, D., Scatassa, M.L., De Filippis, F., Mancuso, I., La Storia, A., Moschetti, G., Settanni, L., 2014. Animal rennets as sources of dairy lactic acid bacteria. Appl. Environ. Microbiol. 80 (7), 2050–2061. https://doi.org/10.1128/AEM.03837-13.

Cruciata, M., Gaglio, R., Scatassa, M.L., Sala, G., Cardamone, C., Palmeri, M., Moschetti, G., La Mantia, T., Settanni, L., 2018. Formation and characterization of early bacterial biofilms on different wood typologies applied in dairy production. Appl. Environ. Microbiol. 84 (4), e02107–e02117. https://doi.org/10.1128/AEM.02107-17.

Da Silva, L.A., Lopes Neto, J.H.P., Cardarelli, H.R., 2019. Safety and probiotic functionality of isolated goat milk lactic acid bacteria. Ann. Microbiol. 69 (13), 1497–1505. https://doi.org/10.1007/s13213-019-01533-z.20.

De Almeida Júnior, W.L.G., Ferrari, Í.D.S., De Souza, J.V., Da Silva, C.D.A., Da Costa, M. M., Dias, F.S., 2015. Characterization and evaluation of lactic acid bacteria isolated from goat milk. Food Control. 53, 96–103. https://doi.org/10.1016/j.foodcont.2015.01.013.

Di Grigoli, A., Francesca, N., Gaglio, R., Guarrasi, V., Moschetti, M., Scatassa, M.L., Settanni, L., Bonanno, A., 2015. The influence of the wooden equipment employed for cheese manufacture on the characteristics of a traditional stretched cheese during ripening. Food Microbiol. 46, 81–91. https://doi.org/10.1016/j.fm.2014.07.008.

Didienne, R., Defargues, C., Callon, C., Meylheuc, T., Hulin, S., Montel, M.C., 2012. Characteristics of microbial biofilm on wooden vats ('gerles') in PDO Salers cheese. Int. J. Food Microbiol. 156 (2), 91. https://doi.org/10.1016/j.ijfoodmicro.2012.03.007.

EFSA, 2018. European Food Safety Authority (EFSA). Panel on Additives and Products or Substances Used in Animal Feed (FEEDAP).

Franciosi, E., Settanni, L., Cavazza, A., Poznanski, E., 2009. Biodiversity and technological potential of wild lactic acid bacteria from raw cows' milk. Int. Dairy J. 19 (1), 3–11. https://doi.org/10.1016/j.idairyj.2008.07.008.

Frétin, M., Martin, B., Rifa, E., Isabelle, V.M., Pomiès, D., Ferlay, A., Montel, M.C., Delbès, C., 2018. Bacterial community assembly from cow teat skin to ripened cheeses is influenced by grazing systems. Sci. Rep. 8 (1), 200. https://doi.org/10.1038/s41598-017-18447-y.

Gaglio, R., Cruciata, M., Di Gerlando, R., Scatassa, M.L., Cardamone, C., Mancuso, I., Sardina, M.T., Moschetti, G., Portolano, B., Settanni, L., 2016a. Microbial activation of wooden vats used for traditional cheese production and evolution of neoformed biofilms. Appl. Environ. Microbiol. 82, 585–595. https://doi.org/10.1128/AEM.02868-15.

Gaglio, R., Couto, N., Marques, C., De Fatima Silva Lopes, M., Moschetti, G., Pomba, C., Settanni, L., 2016b. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int. J. Food Microbiol. 236, 107–114. https://doi.org/10.1016/j.ijfoodmicro.2016.07.020.

Gaglio, R., Francesca, N., Di Gerlando, R., Mahony, J., De Martino, S., Stucchi, C., Moschetti, G., Settanni, L., 2017. Enteric bacteria of food ice and their survival in alcoholic beverages and soft drinks. Food Microbiol. 67, 17–22. https://doi.org/10.1016/j.fm.2017.04.020.

Gaglio, R., Cruciata, M., Scatassa, M.L., Tolone, M., Mancuso, I., Cardamone, C., Corona, O., Todaro, M., Settanni, L., 2019. Influence of the early bacterial biofilms developed on vats made with seven wood types on PDO Vastedda della valle del Belìce cheese characteristics. Int. J. Food Microbiol. 291, 91–103.

Gaglio, R., Busetta, G., Gannuscio, R., Settanni, L., Licitra, G., Todaro, M., 2022. A multivariate approach to study the bacterial diversity associated to the wooden shelves used for aging traditional Sicilian cheeses. Foods 11 (5), 774.

Gargano, V., Sciortino, S., Gambino, D., Costa, A., Agozzino, V., Reale, S., Alduina, R., Vicari, D., 2021. Antibiotic susceptibility profile and tetracycline resistance genes detection in *Salmonella* spp. strains isolated from animals and food. Antibiotics 10, 809. https://doi.org/10.3390/antibiotics10070809.

Guarcello, R., De Angelis, M., Settanni, L., Formiglio, S., Gaglio, R., Minervini, F., Moschetti, G., Gobbetti, M., 2016. Selection of amine-oxidizing dairy lactic acid bacteria and identification of the enzyme and gene involved in the decrease of biogenic amines. Appl. Environ. Microbiol. 82 (23), 6870–6880. https://doi.org/10.1128/AEM.01051-16.

Hernández, D., Cardell, E., Zárate, V., 2005. Antimicrobial activity of lactic acid bacteria isolated from Tenerife cheese: initial characterization of plantaricin TF711, a bacteriocin-like substance produced by Lactobacillus plantarum TF711. J. Appl. Microbiol. 99 (1), 77–84. https://doi.org/10.1111/j.1365-2672.2005.02576.x.

Herreros, M.A., Sandoval, H., González, L., Castro, J.M., Fresno, J.M., Tornadijo, M.E., 2005. Antimicrobial activity and antibiotic resistance of lactic acid bacteria isolated from Armada cheese (a Spanish goats' milk cheese). Food Microbiol. 22 (5), 455–459. https://doi.org/10.1016/j.fm.2004.11.007.

Hossain, Md.I., Sadekuzzaman, M., Ha, S.D., 2017. Probiotics as potential alternative biocontrol agents in the agriculture and food industries: a review. Food Res. Int. 100, 63–73. https://doi.org/10.1016/j.foodres.2017.07.077.

Husson-Kao, C., Mengaud, J., Gripon, J.-C., Benbadis, L., Chapot-Chartier, M.-P., 1999. The autolysis of *Streptococcus thermophilus* DN-001065 is triggered by several food-grade environmental signals. Int. Dairy J. 9 (10), 715–723. https://doi.org/10.1016/S0958-6946(99)00145-4.

Husson-Kao, C., Mengaud, J., Gripon, J.-C., Benbadis, L., Chapot-Chartier, M.-P., 2000. Characterization of *Streptococcus thermophilus* strains that undergo lysis under unfavourable environmental conditions. Int. J. Food Microbiol. 55 (1–3), 209–213. https://doi.org/10.1016/S0168-1605(00)00166-5.

Lortal, S., Di Blasi, A., Madec, M.N., Pediliggieri, C., Tuminello, L., Tanguy, G., Fauquant, J., Lecuona, Y., Campo, P., Carpino, S., Licitra, G., 2009. Tina wooden vat biofilm: a safe and highly efficient lactic acid bacteria delivering system in PDO Ragusano cheese making. Int. J. Food Microbiol. 132 (1), 1–8. https://doi.org/10.1016/j.ijfoodmicro.2009.02.026.

Lortal, S., Licitra, G., Valence, F., 2014. Wooden tools: reservoirs of microbial biodiversity in traditional cheesemaking. Microbiol. Spectr. 2 (1), 2.1.11. https://doi.org/10.1128/microbiolspec.CM-0008-2012.

Lüdin, P., Roetschi, A., Wüthrich, D., Bruggmann, R., Berthoud, H., Shani, N., 2018. Update on tetracycline susceptibility of *Pediococcus acidilactici* based on strains isolated from swiss cheese and whey. J. Food Prot. 81 (10), 1582–1589. https://doi.org/10.4315/0362-028X.JFP-18-160.

Luo, L., Yi, L., Chen, J., Liu, B., Lü, X., 2021. Antibacterial mechanisms of bacteriocin BM1157 against *Escherichia coli* and *Cronobacter sakazakii*. Food Control 123, 107730. https://doi.org/10.1016/j.foodcont.2020.107730.

Makete, G., Aiyegoro, O.A., Thantsha, M.S., 2017. Isolation, identification and screening of potential probiotic bacteria in milk from South African Saanen goats. Probiotics Antimicrob. Proteins. 9 (3), 246–254. https://doi.org/10.1007/s12602-016-9247-5.

Mallia, S., Carpino, S., Corralo, L., Tuminello, L., Gelsonimo, R., Licitra, G., 2005. Effects of aroma profiles of Piacentinu and Ricotta cheese using different tool materials during cheese making. In: Spanier, A.M., Shahidi, F., Parliment, T.H., Mussinan, C., Ho, C.T., Tratras Contis, E. (Eds.), Food flavor and chemistry: explorations into the 21st century. Royal Society of Chemistry, Cambridge, United Kingdom, pp. 23–34.

Mayo, B., Rodríguez, J., Vázquez, L., Flórez, A.B., 2021. Microbial interactions within the cheese ecosystem and their application to improve quality and safety. Foods 10 (3), 602. https://doi.org/10.3390/foods10030602.

Miao, J., Zhou, J., Liu, G., Chen, F., Chen, Y., Gao, X., Dixon, W., Song, M., Xiao, H., Cao, Y., 2016. Membrane disruption and DNA binding of *Staphylococcus aureus* cell induced by a novel antimicrobial peptide produced by *Lactobacillus paracasei* subsp. *tolerans* FX-6. Food Control. 59, 609–613. https://doi.org/10.1016/j.foodcont.2015.06.044.

Monfredini, L., Settanni, L., Poznanski, E., Cavazza, A., Franciosi, E., 2012. The spatial distribution of bacteria in Grana-cheese during ripening. Syst. Appl. Microbiol. 35 (1), 54–63. https://doi.org/10.1016/j.syapm.2011.07.002.

Niederhäusern, S. de, Camellini, S., Sabia, C., Iseppi, R., Bondi, M., Messi, P., 2020. Antilisterial activity of bacteriocins produced by lactic bacteria isolated from dairy products. Foods 9 (12), 1757. https://doi.org/10.3390/foods9121757.

Obioha, P.I., Anyogu, A., Awamaria, B., Ghoddusi, H.B., Ouoba, L.I.I., 2023. Antimicrobial resistance of lactic acid bacteria from Nono, a naturally fermented milk product. Antibiotics 12 (5), 843. https://doi.org/10.3390/antibiotics12050843.

O'Sullivan, L., O'Connor, E.B., Ross, R.P., Hill, C., 2006. Evaluation of live-culture-producing lacticin 3147 as a treatment for the control of *Listeria monocytogenes* on the surface of smear-ripened cheese. J. Appl. Microbiol. 100 (1), 135–143. https://doi.org/10.1111/j.1365-2672.2005.02747.x.

Perin, L., Nero, L., 2014. Antagonistic lactic acid bacteria isolated from goat milk and identification of a novel nisin variant *Lactococcus lactis*. BMC Microb. 14 (1), 36. https://doi.org/10.1186/1471-2180-14-36.

Picon, A., Garde, S., Ávila, M., Nuñez, M., 2016. Microbiota dynamics and lactic acid bacteria biodiversity in raw goat milk cheeses. Int. Dairy J. 58, 14–22. https://doi.org/10.1016/j.idairyj.2015.09.010.

Psoni, L., Kotzamanidis, C., Yiangou, M., Tzanetakis, N., Litopoulou-Tzanetaki, E., 2007. Genotypic and phenotypic diversity of *Lactococcus lactis* isolates from Batzos, a Greek PDO raw goat milk cheese. Int. J. Food Microbiol. 114 (2), 211–220. https://doi.org/10.1016/j.ijfoodmicro.2006.09.020.

Raheel, I., Mohammed, A.N., Mohamed, A.A., 2023. The efficacy of bacteriocins against biofilm-producing bacteria causing bovine clinical mastitis in dairy farms: a new strategy. Curr. Microbiol. 80 (7), 229. https://doi.org/10.1007/s00284-023-03324-x.

Rocha, K.R., Perini, H.F., De Souza, C.M., Schueler, J., Tosoni, N.F., Furlaneto, M.C., Furlaneto-Maia, L., 2019. Inhibitory effect of bacteriocins from enterococci on developing and preformed biofilms of *Listeria monocytogenes*, *Listeria ivanovii* and *Listeria innocua*. World J. Microbiol. Biotechnol. 35 (7), 96. https://doi.org/10.1007/s11274-019-2675-0.

Rojo-Bezares, B., Sáenz, Y., Poeta, P., Zarazaga, M., Ruiz-Larrea, F., Torres, C., 2006. Assessment of antibiotic susceptibility within lactic acid bacteria strains isolated from wine. Int. J. Food Microbiol. 111 (3), 234–240. https://doi.org/10.1016/j.ijfoodmicro.2006.06.007.

Scatassa, M.L., Gaglio, R., Macaluso, G., Francesca, N., Randazzo, W., Cardamone, C., Di Grigoli, A., Moschetti, G., Settanni, L., 2015. Transfer, composition and technological characterization of the lactic acid bacterial populations of the wooden vats used to produce traditional stretched cheeses. Food Microbiol. 52, 31–41. https://doi.org/10.1016/j.fm.2015.06.008.

Schirru, S., Todorov, S.D., Favaro, L., Mangia, N.P., Basaglia, M., Casella, S., Comunian, R., Franco, B.D.G.D.M., Deiana, P., 2012. Sardinian goat's milk as source of bacteriocinogenic potential protective cultures. Food Control. 25 (1), 309–320. https://doi.org/10.1016/j.foodcont.2011.10.060.

Settanni, L., Moschetti, G., 2010. Non-starter lactic acid bacteria used to improve cheese quality and provide health benefits. Food Microbiol. 27 (6), 691–697. https://doi.org/10.1016/j.fm.2010.05.023.

Settanni, L., Moschetti, G., 2014. New trends in technology and identity of traditional dairy and fermented meat production processes: preservation of typicality and hygiene. Trends Food Sci. Technol. 37 (1), 51–58. https://doi.org/10.1016/j.tifs.2014.02.006.

Settanni, L., Di Grigoli, A., Tornambé, G., Bellina, V., Francesca, N., Moschetti, G., Bonanno, A., 2012. Persistence of wild *Streptococcus thermophilus* strains on wooden vat and during the manufacture of a traditional Caciocavallo type cheese. Int. J. Food Microbiol. 155 (1–2), 73–81. https://doi.org/10.1016/j.ijfoodmicro.2012.01.022.

Settanni, L., Gaglio, R., Guarcello, R., Francesca, N., Carpino, S., Sannino, C., Todaro, M., 2013. Selected lactic acid bacteria as a hurdle to the microbial spoilage of cheese: application on a traditional raw ewes' milk cheese. Int. Dairy J. 32 (2), 126–132.

Settanni, L., Busetta, G., Puccio, V., Licitra, G., Franciosi, E., Botta, L., Di Gerlando, R., Todaro, M., Gaglio, R., 2021. In-depth investigation of the safety of wooden shelves used for traditional cheese ripening. Appl. Environ. Microbiol. 87 (23) https://doi.org/10.1128/AEM.01524-21 e01524-21.

Stefańska, I., Kwiecień, E., Jóźwiak-Piasecka, K., Garbowska, M., Binek, M., Rzewuska, M., 2021. Antimicrobial susceptibility of lactic acid bacteria strains of potential use as feed additives—the basic safety and usefulness criterion. Front. Vet. Sci. 8, 687071 https://doi.org/10.3389/fvets.2021.687071.

Stenlid, J., Karlsson, J.O., Hogberg, N., 1994. Intra-specific genetic variation in Heterobasidium annosum revealed by amplification of minisatellite DNA. Mycol. Res. 98, 57e63.

Sucato, A., Vecchioni, L., Savoca, D., Presentato, A., Arculeo, M., Alduina, R., 2021. A comparative analysis of aquatic and polyethylene-associated antibiotic-resistant microbiota in the Mediterranean Sea. Biology 10, 200. https://doi.org/10.3390/biology10030200.

Sudagidan, M., Yemenï CïOğlu, A., 2012. Effects of nisin and lysozyme on growth inhibition and biofilm formation capacity of *Staphylococcus aureus* strains isolated from raw milk and cheese samples. J. Food Prot. 75 (9), 1627–1633. https://doi.org/10.4315/0362-028x.jfp-12-001.

Sun, L., D'Amico, D.J., 2023a. Characterization of microbial community assembly on new wooden vats for use in cheese production. Food Microbiol. 109, 104154 https://doi.org/10.1016/j.fm.2022.104154.

Sun, L., D'Amico, D.J., 2023b. The impact of environmental conditions and milk type on microbial communities of wooden vats and cheeses produced therein. Food Microbiol. 115, 104319 https://doi.org/10.1016/j.fm.2023.104319.

Tatsaporn, T., Kornkanok, K., 2020. Using potential lactic acid bacteria biofilms and their compounds to control biofilms of foodborne pathogens. Biotechnol. Rep. 26, e00477 https://doi.org/10.1016/j.btre.2020.e00477.

Trejo-González, L., Gutiérrez-Carrillo, A.E., Rodríguez-Hernández, A.I., del Rocío López-Cuellar, Ma., Chavarría-Hernández, N., 2022. Bacteriocins produced by lab isolated from cheeses within the period 2009–2021: a review. Probiotics Antimicrob. Proteins 14 (2), 238–251. https://doi.org/10.1007/s12602-021-09825-0.

Vert, M., Doi, Y., Hellwich, K.H., Hess, M., Hodge, P., Kubisa, P., Rinaudo, M., Schué, F., 2012. Terminology for biorelated polymers and applications (IUPAC Recommendations 2012). Pure Appl. Chem. 84 (2), 377–410. https://doi.org/10.1351/PAC-REC-10-12-04.

Vieco-Saiz, N., Belguesmia, Y., Raspoet, R., Auclair, E., Gancel, F., Kempf, I., Drider, D., 2019. Benefits and inputs from lactic acid bacteria and their bacteriocins as alternatives to antibiotic growth promoters during food-animal production. Front. Microbiol. 10, 57. https://doi.org/10.3389/fmicb.2019.00057.

Vitale, M., Galluzzo, P., Buffa, P.G., Carlino, E., Spezia, O., Alduina, R., 2019. Comparison of antibiotic resistance profile and biofilm production of *Staphylococcus aureus* isolates derived from human specimens and animal-derived samples. Antibiotics 8 (3), 97. https://doi.org/10.3390/antibiotics8030097.

Wadhawan, K., Steinberger, A.J., Rankin, S.A., Suen, G., Czuprynski, C.J., 2021. Characterizing the microbiota of wooden boards used for cheese ripening. JDS Comm. 2 (4), 171–176. https://doi.org/10.3168/jdsc.2020-0014.

Wang, K., Zhang, H., Feng, J., Ma, L., Fuente-Núñez, C.D.L., Wang, S., Lu, X., 2019. Antibiotic resistance of lactic acid bacteria isolated from dairy products in Tianjin, China. J. Agric. Food Res. 1, 100006 https://doi.org/10.1016/j.jafr.2019.100006.

Yang, E., Yang, Q., Troemper, B., Zhang, J., 2023. Investigation on bacterial growth and pH in milk after the expiration date. Sci. World J. 2023, 1–9. https://doi.org/10.1155/2023/9982886.

Yi, L., Qi, T., Hong, Y., Deng, L., Zeng, K., 2020. Screening of bacteriocin-producing lactic acid bacteria in Chinese homemade pickle and dry-cured meat, and bacteriocin identification by genome sequencing. LWT–Food Sci. Technol. 125, 109177 https://doi.org/10.1016/j.lwt.2020.109177.