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Antilisterial activity of raw sheep milk from two native Epirus breeds: Culture-dependent identification, bacteriocin gene detection and primary safety evaluation of the antagonistic LAB biota

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ABSTRACT

Raw milk from native small ruminant breeds in Epirus, Greece, is a valuable natural source of autochthonous lactic acid bacteria (LAB) strains with superior biotechnological properties. In this study, two bulk milks (RM1, RM2) from two local sheep yards, intended for traditional Kefalotyri cheese production, were preselected for bacteriocin-like antilisterial activity by in vitro tests. Their antagonistic LAB biota was quantified followed by polyphasic (16S rRNA gene sequencing; IGS for *Enterococcus*; a multiplex-PCR for *Leuconostoc*) identification of 42 LAB (RM1/18; RM2/24) isolates further evaluated for bacteriocin encoding genes and primary safety traits. Representative isolates of the numerically dominant mesophilic LAB were *Leuconostoc mesenteroides* (10) in both RMs, *Streptococcus parauberis* (7) in RM2, and *Lactococcus lactis* (1) in RM1; the subdominant thermophilic LAB isolates were *Enterococcus durans* (8), *E. faecium* (6), *E. faecalis* (3), *E. hirae* (1), *E. hermanniensis* (1), *Streptococcus lutetiensis* (2), *S. equinus* (1) and *S. gallolyticus* (1). Based on their *rpoB, araA, dsr* and *sorA* profiles, six *Ln. mesenteroides* strains (8 isolates) were atypical lying between the subspecies *mesenteroides* and *dextranicum,* whereas two strains profiled with *Ln. mesenteroides* subsp. *jonggajibkimchi* that is first-time reported in Greek dairy food. Two RM1 *E. faecium* strain biotypes (3 isolates) showed strong, enterocin-mediated antilisterial activity due to *entA/entB/entP* possession. One *E. durans* from RM1 possessed *entA* and *entP*, while additional nine RM2 isolates of the *E. faecium/durans* group processed *entA* or *entP* singly. All showed direct (cell-associated) antilisterial activity only, as also both *S. lutetiensis* strains from RM2 did strongly. Desirably, no LAB isolate was β-hemolyrtic, or cytolysin-positive, or possessed *vanA, vanB* for vancomycin resistance, or *agg, espA, hyl,* and *IS16* virulence genes. However, all three *E. faecalis* from RM2 possessed *gelE* and/or *ace* virulence genes. In conclusion, all *Ln. mesenteroides* strains, the two safe, enterocin A-B-P-producing *E. faecium* strains, and the two antilisterial *S. lutetiensis* strains should be validated further as potential costarter or adjunct cultures in Kefalotyri cheese. The prevalence of α-hemolytic pyogenic streptococci in raw milk, mainly *S. parauberis* in RM2, requires consideration in respect to subclinical mastitis in sheep and the farm hygiene overall.

1. Introduction

The microbiota of raw milk is quite complex, consisting of a great variety of bacterial, yeast and mould genera and species (Quigley et al., 2013). The application of powerful culture-dependent and high throughput sequencing techniques has further increased the microbial taxa detected by conventional methods (Oikonomou et al., 2020; Parente et al., 2020; Tilocca et al., 2020). Many of the species occurring in raw milk are beneficial biotechnologically and/or for human and animal health, whereas many others are spoilage or pathogenic microorganisms. Specifically, certain lactic acid bacteria (LAB) species of the genera *Lactococcus, Streptococcus, Leuconostoc, Enterococcus* and *Pediococcus,* and of the former genus *Lactobacillus* reclassified as *Lactobacillus sensu stricto, Lacticaseibacillus, Lactiplantibacillus, Latilactobacillus* and *Levilactobacillus* (Zheng et al., 2020), occur commonly in raw cow, buffalo, sheep or goat milks (Quigley et al., 2013) and contribute greatly to the production of traditional raw milk cheeses (Montel et al., 2014; Coelho et al., 2022). Certain *Staphylococcus, Corynebacterium, Brevibacterium,* and *Propionibacterium* species found in raw milk also exert beneficial cheese ripening effects or they are essential for the production

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of specific cheese types (Quigley et al., 2013). Conversely, psychrotrophic, gram-negative bacteria of the families *Pseudomonaceae* and *Enterobacteriaceae* and gram-positive sporoforming *Bacillus* and *Clostridium* spp. are important milk and cheese spoilers (Doyle et al., 2015; Yuan et al., 2019; Martin et al., 2023), albeit desirable aromatic activities may be exerted in raw milk cheeses by few *Enterobacteriaceae*, mainly *Hafnia alvei* (Irlinger et al., 2012; Montel et al., 2014). Pathogenic bacteria most frequently encountered in raw animal milks intended for cheese production include *Listeria monocytogenes, Salmonella*, enterohaemorrhagic *Escherichia coli, Campylobacter*, and certain *Staphylococcus* and *Streptococcus* species associated with milk from animals suffering mastitis, mainly *Staphylococcus aureus* and *Streptococcus uberis* (Kousta et al., 2010; Quigley et al., 2013; Gonzales-Barron et al., 2017; Williams et al., 2023).

Milk in healthy udder cells is not sterile, as thought to be in the early 1980's; instead, all teat milks contain a primary animal species-dependent and possibly breed-dependent antagonistic maternal gut biota transferred to the mammary gland (Quigley et al., 2013; Oikonomou et al., 2020; Martin et al., 2023). Then this biota is mixed with diverse beneficial or harmful microorganisms from various environmental contamination sources during and after milking, including the teat apex, milking equipment, air, water, feed, grass, soil and other farm or plant sites (Kousta et al., 2010; Quigley et al., 2013; Parente et al., 2020). Eventually, during raw milk cheese processing, all antagonistic LAB present in raw milk, many of which produce bacteriocins additionally to organic acids (Rodriguez et al., 2000; Perin et al., 2012; Yoon et al., 2016), may contribute to control growth of spoilage and pathogenic bacteria, particularly of *L. monocytogenes* (Montel et al., 2014; Coelho et al., 2022), regardless of their aforementioned origin. However, the natural synergistic antilisterial activity of the complex microbial (LAB) consortia that often is enough strong to suppress *L. monocytogenes* growth in raw milk (Lianou and Samelis, 2014) or raw milk cheese (Millet et al., 2006; Montel et al., 2014), is minimized by pasteurization (Quigley et al., 2013) or reduced by thermization (Lianou and Samelis, 2014) required to assure cheese safety. Therefore, the re-introduction in pasteurized or thermized cheese milks of selected starter, co-starter or adjunct LAB strains, which apart from their in situ antilisterial activity may enhance the flavor and many other sensory qualities of the resultant cheese products by their biochemical activities associated with carbohydrate, citrate, protein and amino acid catabolism (Wouters et al., 2002; Anastasiou et al., 2022; Coelho et al., 2022), can compensate for the removal of the indigenous antagonistic LAB biota inactivated by pasteurization (Quigley et al., 2013) or even thermization (Samelis et al., 2009; Tilocca et al., 2020) of raw milk.

In Greece and particularly in Epirus, sheep milk of native breeds, Boutsiko, Karamaniko, Frisarta, Karagouniko and others, is the primary milk used for traditional cheese production (Fotou et al., 2011; Kondyli et al., 2012; Skoufos et al., 2017), most often after mixing with 10–30 % goat milk, pasteurization or mild thermization, and then addition of natural or commercial starters, depending on the cheese variety and the processor's practical experience (Samelis et al., 2009; Voidarou et al., 2011; Vandera et al., 2019). So far numerous surveys have been conducted to evaluate the hygienic quality of bulk/tank Greek/Epirus raw sheep and goat milks, with emphasis on total microbial and somatic cell counts, the presence of bacterial pathogens, primarily *S. aureus* (Morgan et al., 2003; Solomakos et al., 2009; Fotou et al., 2011; Alexopoulos et al., 2011; Zdragas et al., 2015; Pexara et al., 2016; Malissiova et al., 2017; Angelidis et al., 2020; Lianou et al., 2021a, 2021b, 2022), and occasionally on microbiological differences between sheep breeds (Kondyli et al., 2012; Skoufos et al., 2017). In contrast, published studies on the microbial ecology of raw sheep or goat milks with emphasis on the isolation, identification and biotechnological characterization of indigenous LAB strains are still limited in Greece (Samelis et al., 2009; Chanos and Williams, 2011; Parapouli et al., 2013), inclusively of few recent studies that have characterized some LAB isolates from raw sheep milk as the first processing step during artisanal Feta, Kefalograviera or Graviera raw milk cheese production (Tsigkrimani et al., 2022a, 2022b; Apostolakos et al., 2023; Psomas et al., 2023).

In this study, the natural antilisterial activity of raw milk from two local sheep yards was evaluated. Bulk milks showing bacteriocin-mediated (Bac+) activity were prescreened by simple challenge tests. Afterward, the indigenous LAB biota occurring in the selected antagonistic raw milk batches was identified, characterized for bacteriocin production and evaluated for primary safety traits by culture-dependent molecular methods. The final aim was to obtain antagonistic (Bac+) strains of diverse LAB species occurring in raw sheep milk, in view of their commercial application as co-starter/protective adjunct cultures in traditional Kefalotyri cheese production from thermized milk, i.e., in replacement of the indigenous antagonistic LAB types inactivated by thermization.

2. Materials and methods

2.1. Raw milk samples

Five raw milk samples (RM1-RM5; 1 l each) representing five individual daily farm milk productions from two sheep yards with mixed native Karamaniko and Karagouniko breeds (RM1, RM3 and RM5: Filippiada yard; RM2 and RM4: Dryofyto yard) crossbred in the area of Arta, Epirus, Greece, were studied. RM samples were aseptically collected by pouring in presterilized Duran bottles before Kefalotyri cheese processing in our collaborating plant (Skarfi E.P.E., Pappas Bros. Traditional Dairy, Filippiada, Epirus, Greece) and transported to the applied microbiology laboratory of the Dairy Research Department (Katsikas, Ioannina, Epirus, Greece) in insulated ice boxes within 45 min after sampling. RM1 and RM2 samples were 'winter' sheep milks collected in January, whereas RM3, RM4 and RM5 were 'summer' sheep milks collected in June. RM samples were mixed thoroughly in their original containers and analyzed promptly after receipt. The entire workflow chart followed during this study is illustrated in Fig. 1 and the analytical methods of RM used in sequence are described in detail in the Sections 2.2–2.4 below.

2.2. Evaluation of the antilisterial activity of raw milk samples by well diffusion assay tests

All five RM batches were tested for direct in situ antilisterial activity before (0-h) and after (48-h) incubation at 37 °C, in general accordance with the procedures described by Vandera et al. (2018). Briefly, the modified well diffusion assay of Lianou and Samelis (2014) and *L. monocytogenes* no.10 inocula (2 % v/v) as indicator strain in TSAYE plates with opened (6-mm diameter) wells were used for direct testing of the fresh or cultured RM samples, as illustrated by Vandera et al. (2018). Strain no.10, originally described by Ralovich (1989) as avirulent in Anton's and mouse tests, is a meat strain of *L. monocytogenes* (serotype 4ab) of high bacteriocin (enterocin) sensitivity but moderate acid sensitivity; therefore, it is routinely used as a highly convenient target and/or indicator *Listeria* strain in our dairy studies, too (Samelis et al., 2009; Vandera et al., 2018, 2020).

The RM samples were first well-assayed as they were undiluted to assess the antilisterial potential of the original levels and types of natural microbial contaminants in raw milk (0-h). Next, the 48-h incubation period served as a 'culture enrichment' to enhance the prevalence of indigenous antagonistic LAB strains (Vandera et al., 2018) in the naturally fermented/acidified samples at 37 °C. Originally, this 'culture enrichment' technique was applied for enhancing the selection of indigenous enterocin-producing (Ent+) *Enterococcus* spp. in thermized milk (TM; 63 °C; 30 s) samples with reduced numbers (<10–150 CFU/mL) of coliform and other gram-negative bacteria before incubation (Vandera et al., 2018). Because the present raw sheep milks were expected to contain numerous undesirable gram-negative bacteria apart from LAB contaminants, two series of 20-mL samples from each RM

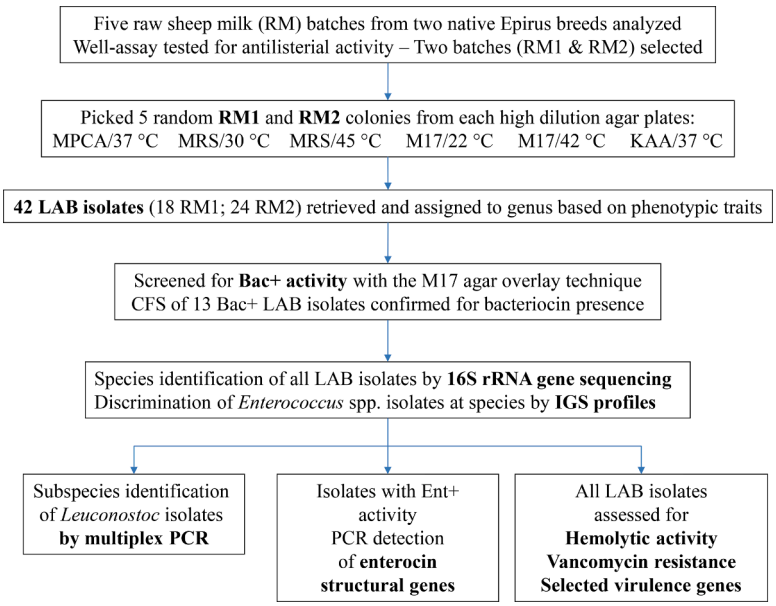

Fig. 1. Workflow chart followed for the isolation, culture-dependent identification, bacteriocin gene detection and primary safety evaluation of 42 LAB isolates from two batches (RM1 and RM2) of fresh raw sheep milk from two native Epirus breeds which were found to retain a strong to moderate in vitro antilisterial activity after a natural fermentation/culture enrichment step at 37 °C for 48 h, without or with added 4 % salt, followed by adjustment of their pH at 6.0-6.2 and heating at 80 °C for 15 min to inactivate viable cells.

batch were challenged at 0 and 48 h, the first series without and the second with added 4 % NaCl. Salt addition aimed to favor growth of indigenous mesophilic LAB, such as wild lactococci, leuconostocs, lactobacilli, and enterococci capable of growing well in 4 %, 6.5 % and/or 8 % salt in vitro (Vandera et al., 2019; Sameli et al., 2021; Samelis and Kakouri, 2022), in raw milk and/or in the well assays during incubation. All 'culture-enriched' curdled RM samples were tested after a 1:1 dilution with Ringer to facilitate diffusion around the wells. In all cases, the inhibitory activity of the 'viable' (acidic; non-heated) RM samples was comparatively evaluated with the remaining activity in the samples after neutralization of the milk acidity by adjusting the pH at 6.0-6.2 with 1N NaOH, followed by total vegetative cell inactivation by heating at 80 °C for 15 min before pouring 60 μL in the wells. Each RM sample type was well-assayed in duplicate. All TSAYE plates were kept at 4 °C for 2-3 h for milk sample diffusion, incubated at 30 °C for 18 h, and examined for the development of inhibition zones of *L. monocytogenes* growth surrounding the wells on the next day (Lianou and Samelis, 2014).

2.3. Enumeration, isolation and basic phenotypic characterization of the indigenous LAB biota present in raw milk samples showing bacteriocin-mediated antilisterial activity

After receipt (0-h), all RM batch samples were in parallel analyzed microbiologically and for pH, according to the procedures described by Samelis et al. (2009). All diluents, microbiological agar media, and supplements were purchased from Neogen Culture Media (Heywood, UK), except of the M17 agar (Biolife, Italiana, S.r.l., Milano, Italy). For the purposes of this study, Milk Plate Count agar (MPCA) incubated at 37 °C for 72 h, de Man, Rogosa, Sharpe (MRS) agar incubated at 30 °C for 72 h or at 45 °C for 48 h, M17 agar incubated at 22 °C for 72 h or at 42 °C for 48 h, and Kanamycin Aesculin Azide (KAA) agar incubated at 37 °C for 48 h were used for the enumeration of the total mesophilic and total thermophilic LAB populations present in the RM samples, followed by isolation of the most prevalent LAB types grown on duplicate plates of each of the above agar media (Samelis et al., 2009). Of note, KAA agar used to selectively enumerate enterococci also supports growth of kanamycin-resistant, esculin-positive *Lactiplantibacillus* spp. when present in milk (Samelis et al., 2009) or cheese (Samelis and Kakouri, 2022) samples.

In the course of this study, only two RM batches that inhibited growth of *L. monocytogenes* no. 10 in all sequent well-assays described in Section 2.2 (viz. Results), were analyzed further. So, after enumeration, 60 presumptive LAB colonies (30 from each inhibitory RM batch; 5 from each agar medium) were randomly picked from one high dilution MPCA/37 °C, MRS/30 °C, MRS/45 °C, M17/22 °C M17/42 °C; KAA/37 °C agar plate, by following the same isolation protocol we applied in previous milk or cheese LAB ecology studies (Vandera et al., 2018, 2019; Samelis and Kakouri, 2022). Depending on their isolation medium, colonies were grown in MRS or M17 broth at 30 °C or 37 °C, purified by streaking on MRS or M17 agar plates, checked for their Gram-staining, catalase and oxidase reactions to separate the LAB (Gram-positive, catalase-negative) isolates, and kept in MRS broth supplemented with 20 % (w/v) glycerol (Merck, Darmstadt, Germany) at -30 °C (Samelis and Kakouri, 2022). For the purposes of this study, only the LAB isolates obtained from RM batches that showed in vitro bacteriocin-mediated antilisterial activity in the aforementioned well diffusion assays (Section 2.2) were characterized further (Fig. 1): initially, they were differentiated in main LAB groups at the genus level by basic phenotypic criteria (cell-shape by phase contrast microscopy; CO_2 from glucose; NH_3 from arginine hydrolysis; growth at 10 °C and 45 °C; growth in 4 % and 6.5 % salt; growth on KAA agar), performed according to Samelis and Kakouri (2022), followed by their identification at the species by genotypic methods (Fig. 1).

2.4. Molecular identification and characterization of the indigenous raw milk LAB isolates

2.4.1. Reference-control LAB strains

For the genomic identification and characterization analyses, the following *Enterococcus* reference strains were used as positive controls in the molecular assays, as appropriate: (i) *E. faecium* KE82 (Greek Graviera cheese isolate; autochthonous, safe; multiple-enterocin (m-Ent+) producer: *entA/entB/entP*); (ii) *E. durans* KE100 (Graviera cheese isolate; autochthonous, safe; single-enterocin (Ent+) producer: *entP*); (iii) *E. durans* KE108 (Graviera cheese isolate; autochthonous, m-Ent+ producer: *entP/bac31;* found to uncommonly possess cytolysin (*cyl*) gene);

(iv) *E. faecalis* GL320 (Greek Galotyri PDO cheese isolate; autochthonous; β-hemolytic; produces cytolysin, *cylL*$_L$+); (v) *E. faecalis* GL322 (Greek Galotyri PDO cheese isolate; autochthonous; α-hemolytic; *cyl*+); (vi) *E. faecalis* ATCC 29212^{TM} (global reference strain; virulent; possesses *gelE* and *ace*); (vii) *E. faecium* 315VR (clinical/human Greek isolate; vancomycin-resistant; *vanA*+). More details on the above *Enterococcus* strains (i.e., origin, mode of antilisterial activity, GenBank accession numbers of the indigenous Greek strains from our laboratory collection) are given by Vandera et al. (2020) and Tsanasidou et al. (2021). Additionally, three reference *Leuconostoc mesenteroides* strains isolated from traditional Greek cheeses, *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0231; *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0493, and *Ln. mesenteroides* subsp. *mesenteroides* ACA-DC 0750, kindly provided by Professor E. Tsakalidou, Laboratory of Dairy Research, Agricultural University of Athens (MTA; 10-02-2022), were used as control strains for the assignment of the RM isolates identified as *Ln. mesenteroides* to the subspecies level.

2.4.2. Identification of LAB isolates by 16S rRNA sequencing, IGS, and multiplex PCR analyses

LAB isolates were identified at the species by 16S rRNA sequencing analyses (Fig. 1), according to the analytical procedures described by Sameli et al. (2021). The species identification of the LAB isolates phenotypically assigned to the genus *Enterococcus* was first determined by intergenic spacer (IGS) analyses, according to Vandera et al. (2020), while the grouping and 16S rRNA identification of heterofermentative LAB isolates as *Ln. mesenteroides* was later confirmed and extended toward their subspecies identification (Fig. 1), according to Ricciardi et al. (2020). The primers used for the above PCR and multiplex PCR analyses are listed in Table 1.

Briefly, the selected isolates were subcultured overnight in MRS broth at 30 °C and genomic DNA was extracted according to

Table 1
List of primers used for PCR and multiplex PCR in this study

Gene	Primer	Sequence (5'-3')	Amplicon size (bp)	Annealing Temperature (°C)	Refs.[1]
16s rRNA Sanger Sequencing					
	8F	AGAGTTTGATCCTGGCTCAG	1500	60	https://help.ezbiocloud.net/16s-rrna-and-16s-rrna-gene/
	1492R	CGGTTACCTTGTTACGACTT			
	515FB	GTGYCAGCMGCCGCGGTAA			
IGS					
IGS	R16-1	GGCTGGATCACCTCCTTTCT		60	Vandera et al. (2020)
	R23-2R	TCCGGGTACTTAGATGTTTC			
Potential virulence genes					
agg	Agg-F	AAGAAAAAGAAGTAGACCAAC	1553	53	Tsanasidou et al. (2021)
	Agg-R	AAACGGCAAGACAAGTAAATA			
ace	Ace-F	CAGGCCAACATCAAGCAACA	125	65	
	Ace-R	GCTTGCCTCGCCTTCTACAA			
espA	EspA-F	TTTGGGGCAACTGGAATAGT	407	53	
	EspA-R	CCCAGCAAATAGTCCATCAT			
IS16	IS16-F	CATGTTCCACGAACCAGAG	547	55	
	IS16-R	TCAAAAAGTGGGCTTGGC			
hyl	Hyl-F	ACAGAAGAGCTGCAGGAAATG	276	58	
	Hyl-R	GACTGACGTCCAAGTTTCCAA			
gelE	GelE-F	CGAAGTTGGAAAAGGAGGC	372	50	
	GelE-R	GGTGAAGAAGTTACTCTGA			
cytlL$_L$	CytlL$_L$-F	GGCGGTATTTTTACTGGAGTA	248	53	
	CytlL$_L$-R	CCTACTCCTAAGCCTATGGTA			
Vancomycin resistance-associated genes					
vanA	VanA1	GCTGCGATATTCAAAGCTCA	545	52	Tsanasidou et al. (2021)
	VanA2	CAGTACAATGCGGCCGTTA			
vanB	VanB1	ATGGGAAGCCGATAGTCTC	368	52	
	VanB3	GTTACGCCAAAGGACGAAC			
Enterocin and bacteriocin genes					
entA	entA-F	GGTACCACTCATAGTGGAAA	138	58	Vandera et al. (2018)
	entA-R	CCCTGGAATTGCTCCACCTAA			
entB	entB-F	CAAAATGTAAAAGAATTAAGTACG	201	56	
	entB-R	AGAGTATACATTTGCTAACCC			
entP	entP-F	GCTACGCGTTCATATGGTAAT	87	54	
	entP-R	TCCTGCAATATTCTCTTTAGC			
entAS-48	entAS-48-F	GAGGAGTATCATGGTTAAAGA	318	56	
	entAS-48-R	ATATTGTTAAATTACCAA			
entL50A	entL50A-F	ATGGGAGCAATCGCAAAATTA	105	55	
	entL50A-R	TTTGTTAATTGCCCATCCTTC			
entL50B	entL50B-F	Identical to entL50A-F	247	51	
	entL50B-R	TAGCCATTTTTCAATTTGATC			
bac31	bac31-F	TATTACGGAAATGGTTTATATTGT	122	52	
	bac31-R	TCTAGGAGCCCAAGGGCC			
Identificaton of *Leuconostoc mesenteroides* at the subspecies level					
rpoB	rpob-F	GTCCGCATTGATCGCACGC	952	60	Ricciardi et al. (2020)
	rpob-R	CACCCGGTCCAAGAGCTGAC			
araA	L-ara-F	TTTGGCTGGACGGTTGACT	744		
	L-ara-R	TGTTGTGTGATGTCCGCCAC			
dsr	dextran-F	TGGCACCATTACCATAACGAACT	549		
	dextran-R	TGCCAGCAGTCGATCAATATGG			
sorA	PTS-sorb-F	GTGCCTTACTCCCCTGTGTAG	253		
	PTS-sorb-R	TCCTCGTCTTCCTCATCATCGT			

[1] Previous studies from our laboratory cited herein have used and listed the same groups of primers by reporting the original reference articles by other workers from which the primer information was gained.

Tsafrakidou et al. (2021). The genomic DNA was used as template for the amplification of the V1 – V9 region of the 16S rRNA gene (1500 bp) using the primers 8F/1492R, and for the IGS region using the primers R16-1/R23-2R (Table 1). All amplification reactions were prepared using the Kapa Taq PCR kit (Kapa Biosystems, Boston Massachusetts, USA) according to the manufacturer instructions using 50 ng of template DNA and a reaction volume of 50 μl. PCR was performed in the DNA Engine Peltier Thermal Cycler (BioRad) using the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 1 min followed by a final extension at 72 °C for 2 min. The amplicons obtained with the 8F/1492R primers were purified using the PCR clean-up Gel extraction kit (Macherey-Nagel, Düren, Germany) and Sanger sequencing was performed using the specific primers 8F, 515FB and 1492R (Table 1) by CeMIA (Larissa, Greece). Sequencing trace files were analysed and assembled into consensus sequences using the GEAR Genome analysis server (gear-genomics.com) (Rausch et al., 2020). Taxonomic classification of the 16S sequence was performed using the GenBank's BLAST program at the NCBI website.

For the IGS analyses, PCR products obtained with the R16-1/R23-2R primers (Table 1) were separated in 1.2 % agarose gel stained with ethidium bromide. *E. faecium* KE82, *E. durans* KE100 and *E. faecalis* GL322 were used as reference (control) strains for confirming the three species differentiation and characterization within all *Enterococcus* spp. isolates from RM samples.

The *Ln. mesenteroides* strains isolated from RM were identified at subspecies using a multiplex-PCR assay (presence/absence of the genes: *rpoB*, L-arabinose isomerase, *araA;* dextransucrase, *dsr*; PTS-sorbose transporter subunit IIC, *sorA*), according to Ricciardi et al. (2020). Specifically, PCR-amplifications were performed using 25 ng of total bacterial DNA, 1 μM of *rpoB* primers, 0.5 μM of *araA* primers, 0.3 μM of *dsr* primers and 0.1 μM of *sorA* primers (Table 1) in 25 μl reaction mixtures using the Kapa Taq PCR kit (Kapa Biosystems), according to the manufacturer instructions. PCR was performed in the DNA Engine Peltier Thermal Cycler (BioRad) using the following steps: 5 min at 95 °C, 30 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 60 s, extension at 72 °C for 90 s and a final extension of 10 min at 72 °C. PCR products were separated in 1.2 % agarose gel stained with ethidium bromide.

2.4.3. Evaluation of the indigenous raw milk LAB isolates for Bac+ antilisterial activity

All LAB isolates were further screened for direct antilisterial activity against the target strain *L. monocytogenes* no.10 by the simple agar overlay technique on M17 agar plates at 30 °C overnight, as described by Vandera et al. (2018). For the isolates causing clear growth inhibition zones, a bacteriocin-like antilisterial activity was confirmed by the well diffusion assay conducted as described in Section 2.2 for RM; in the pure culture assays, however, the wells on the fresh TSAYE lawns of *L. monocytogenes* no.10 were filled with 60 μl of cell-free supernatant (CFS) of each tested LAB strain following growth in MRS or M17 broth at 30 °C for 24 h. The CFS samples were prepared according to Vandera et al. (2020) and tested further after adjustment of their pH at 6.0-6.2 to eliminate the (lactic) acid inhibitory effects, followed by treatment with proteolytic enzymes (proteinase K, α-chymotrypsin, trypsin; each enzyme was added to CFS at a final concentration of 1 mg/mL and the samples were incubated at 37 °C for 3–24 h) to ensure loss of the bacteriocin-mediated activity. The type of each proteolytic enzyme, all purchased from Sigma (Sigma Aldrich Chemie GmbH, Steinheim, Germany), are given by Vandera et al. (2020). Because all RM strains confirmed for CFS antilisterial Bac+ activity by the well assay were enterococci, all *Enterococcus* spp. isolates from the RM1 and RM2 samples were tested comparatively for the possession of common structural enterocin genes in their genome, according to Vandera et al. (2018, 2020).

Briefly, the primer pairs for enterocins A, B, P, L50A, L50B, AS-48, and Bacteriocin 31 were used (Table 1). *Enterococcus faecium* KE82, *E. durans* KE100 and *E. durans* KE108 were used as positive controls for the detection of *entA, entB, entP*, and *bac31* genes, as reported for each strain in 2.4.1 above (Vandera et al., 2020). The Kapa Taq PCR kit (Kapa Biosystems) was used for PCR amplification, according to the manufacturer instructions, using 25 ng of total bacterial DNA in 25 μl reaction mixtures. PCR was performed in the DNA Engine Peltier Thermal Cycler (BioRad) using the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, various annealing temperatures (viz. Table 1) for 30 s, extension at 72 °C for 1 min followed by a final extension at 72 °C for 2 min. Amplification products were separated in 1.2 % agarose gel stained with ethidium bromide. PCR positive results were verified by Sanger sequencing, as follows: amplicons obtained with the specific primers for enterocin A, enterocin B and enterocin P from one representative m-Ent+ *E. faecium* strain with strong activity (see Results), were purified using the PCR clean-up Gel extraction kit (Macherey-Nagel) and Sanger sequencing was performed using the primers entA-F, entB-F and entP-R (Table 1) by CeMIA. Sequences obtained were analyzed using the GenBank's BLAST program at the NCBI website.

2.4.4. Safety evaluation of the indigenous raw milk LAB isolates

Next, all LAB colonies were tested for their safety status, which included (i) *in vitro* testing for β-hemolysis and PCR-detection of *cylL*$_L$ (cytolysin), according to Vandera et al. (2018, 2020); (ii) PCR-detection of *vanA* and *vanB* genes for vancomycin resistance, and (iii) PCR-detection of the *agg* (aggregation substance), *ace* (accessory colonization factor), *espA* (enterococcal surface protein), *IS16* (transportable element), *hyl* (hyaluronidase) and *gelE* (gelatinase) virulence genes, according to Tsanasidou et al. (2021). The primer pairs used are listed in Table 1. Positive control (reference) strains were *E. faecalis* GL320 for β-hemolysis and *cylL*$_L$, *E. faecalis* ATCC 29212^{TM} for *gelE, ace* and *cylL*$_L$ and *E. faecium* 315VR for *vanA*. PCR-amplifications were carried out using 25 ng of total bacterial DNA in 25 μl reaction mixtures and the Kapa Taq PCR kit (Kapa Biosystems), according to the manufacturer instructions. PCR was performed in the DNA Engine Peltier Thermal Cycler (BioRad) using the following conditions: initial denaturation at 95 °C for 3 min, 35 cycles of denaturation at 95 °C for 30 s, various annealing temperatures (viz. Table 1) for 30 s, extension at 72 °C for 1 min followed by a final extension at 72 °C for 2 min. Amplification products were separated in 1.2 % agarose gel stained with ethidium bromide.

3. Results and discussion

3.1. Variations in the antilisterial activity of raw sheep milk samples before and after their natural fermentation at 37 °C, with or without 4 % salt supplementation

Among the five fresh RM batch (pH 6.6 ± 0.1) samples tested without supplying salt, only RM4 (pH 6.5) showed slight growth inhibitory activity of *L. monocytogenes* no.10, which was retained in the wells with the fresh RM4 supplied with 4 % salt (Table 2). The fresh/salted RM1 also was weakly inhibitory. Incubation of all RM samples at 37 °C for 48 h enhanced a natural fermentation/ acidification which was more pronounced, in terms of milk pH drop, in the unsalted (pH 4.0–4.4) than in the salted (pH 4.2–4.8) 'viable' curdled RM samples. All of them inhibited *L. monocytogenes* growth when they were poured undiluted into the wells with the aid of sterile cut-edged plastic tips; this inhibition was attributed to the high density of viable (LAB) cells, acid production by them and probably to other potent natural antimicrobials present in RM, such as lactoperoxidase. When, however, the naturally acidified/curdled RM samples were diluted (1:1) with sterile Ringer to improve diffusion, only RM1, RM2 and RM4 continued to manifest a direct antilisterial activity; of note, the salted 'fermented' RM1 was more inhibitory than the unsalted 'fermented' RM1, and *vice versa* for RM2

Table 2
Antilisterial activity of five raw milk batches collected from two sheep yards located in Epirus, as detected by a series of sequent well diffusion assays against the indicator strain *Listeria monocytogenes* no.10.

Raw milk batch	Raw milk treatment before well assay testing [1]					
	Fresh/Non-acidic (No treatment)		Fermented/Acidic		Fermented/ Neutralized/Boiled	
	No salt	4 % NaCl	No salt	4 % NaCl	No salt	4 % NaCl
RM1	-	(+)	+	++	(+)	++
RM2	-	-	++	+	-	+
RM3	-	-	-	-	-	-
RM4	(+)	(+)	+	+	-	-
RM5	-	-	-	-	-	-

-, No inhibition zone; (+), weak-faint inhibition zone (<2 mm); +, clear inhibition zone (2–5 mm); ++, strong inhibition zone (>5 mm).

[1] Fresh/Non-acidic: Raw milk (RM) samples were tested as they were (untreated), without pre-incubation (0-h); Fermented/Acidic: RM samples were tested after incubation at 37 °C for 48 h and dilution (50:50) with sterile Ringer solution; Fermented/Neutralized/Boiled: The fermented/acidic samples were tested after their pH was adjusted to pH 6.0-6.2 followed by boiling at 80 °C for 15 min to inactivate viable microbial (LAB) cells. All challenge treatments were done without or with 4 % salt addition in the fresh raw milk samples taken from each batch.

(Table 2). Eventually, when the 'culture-enriched' RM samples were neutralized (ca. pH 6.0) to remove acid effects and then heated (80 °C; 15 min) to inactivate the viable (LAB) cells and activity of endogenous RM enzymes, only RM1 (mainly) and the salted RM2 'winter' batch samples retained a bacteriocin-like inhibitory activity against *L. monocytogenes* no.10 (Table 2). Similar results of fluctuating antilisterial activity were obtained for the control (uninoculated) sheep/goat (90:10) RM or TM samples during previous studies assessing the in situ nisin A or enterocin A-B-P activity of our indigenous bioprotective strains *Lc. lactis* subsp. *cremoris* M104 and *E. faecium* KE82, respectively (Lianou and Samelis, 2014; Vandera et al., 2018). In fact, only one neutralized/heated TM control sample had retained good enterocin activity (Vandera et al., 2018), a finding that prompted us to add 4 % salt in the present RM samples without LAB inoculation. Correspondingly, the results in Table 2 suggest that RM1 harbored numerous salt-tolerant, Bac+ LAB, probably enterococci, whereas the increased antilisterial activity of the acidic/viable RM2 samples was probably due to salt-sensitive indigenous LAB types. Of note, the aforementioned challenge studies had used spring or summer milks; this is the first time we challenged winter RMs for total direct versus bacteriocin-like antilisterial activity.

3.2. Prevalence of coccoid LAB genera in raw sheep milks showing in situ bacteriocin-like activity

Thus, only the two winter batches, RM1 (pH 6.6) and RM2 (pH 6.7) were studied further (Fig. 1). Total bacterial populations grown on the MPCA/37 °C, MRS/30 °C, MRS/45 °C, M17/22 °C, M17/42 °C and KAA/37 °C agar plates were 6.27, 5.29, 3.67, 7.11, 5.90, and 3.88 log CFU/mL and 5.16, 5.25, 3.74, 6.18, 5.20, and 4.11 log CFU/mL for the RM1 and RM2, respectively. Of totally 60 presumptive LAB colonies, (30/batch; 5/agar medium) picked from the above plates, 42 LAB isolates, 18 from RM1 (coded KFM1-KFM30) and 24 from RM2 (KFM31-KFM60) were obtained (Fig. 1). The remaining 18 isolates were non-LAB; 15 of them were gram-negative, oxidase-positive (*Pseudomonas*-like) or oxidase-negative (*Enterobacteriacae*-like) bacteria predominantly isolated from the M17/22 °C or M17/42 °C agar plates of both RM batches only, while the remaining three isolates (2 RM1 from MPCA/37 °C and 1 RM2 from M17/42 °C) were gram-positive, catalase-positive cocci. In contrast, only LAB colonies were isolated from all KAA and MRS agar plates, irrespective of the incubation temperature. Thus, the natural microbiota of the two winter RM batches comprised mainly psychrotrophic gram-negative spoilage bacteria which grew predominantly on M17/22 °C agar, particularly in RM1, followed by mesophilic LAB. Thermophilic LAB (MRS/45 °C) and enterococci (KAA) were subdominant in both RM batches.

The phenotypic characterization confirmed the above basic observations and further revealed that all 42 LAB isolates were cocci; no lactobacilli were detected (Table 3). They were differentiated in five distinct phenotypic groups, A-E, assigned to the genera *Leuconostoc* (Group A; 10 isolates), *Enterococcus* (Group B; 19 isolates), mesophilic, arginine-positive *Lactococcus* or *Streptococcus* (Group C; 7 isolates), thermophilic *Streptococcus* (Group D; 4 isolates), whereas two atypical homofermentative LAB isolates included in group E, KFM8 from RM1 and KFM37 from RM2, lied between streptococci, lactococci and/or arginine-negative enterococci (Table 3). There was an even distribution of the isolates in each LAB group between the two RM batches, except of the seven mesophilic, arginine-positive *Lactococcus* or *Streptococcus* (Group C) solely isolated from RM2 batch (Table 3). Of note, all ten *Leuconostoc* spp. (Group A) were isolated from MRS/30 °C (mainly) or MPCA/37 °C whereas all seven RM2 isolates in Group C were recovered from lactose-containing agars only. Conversely, all *Enterococcus* spp. were isolated from KAA (10/10) and MRS/45 °C (8/10) agars, except of one typical enterococcal strain (KFM6) and both atypical strains in group E isolated from MRS/30 °C plates. Unsurprisingly, all four thermophilic *Streptococcus* (Group D) were isolated from agar media plates incubated at 37–45 °C, except from KAA (Table 3).

Altogether the above results indicated that leuconostocs (Group A) were the most prevalent LAB in RM1 batch, whereas mesophilic lactococci or streptococci (Group C) intermixed with leuconostocs (Group A) prevailed in RM2 batch. Enterococci (Group B) were subdominant in both RM batches; however, their net isolation frequency was the highest (45.2 % of the LAB isolates) due to the high enterococcal selectivity of the KAA and MRS/45 °C agars (Table 3). These findings are generally consistent with the presence of *Lactococcus, Streptococcus, Leuconostoc* and *Enterococcus* in raw sheep milk (Quigley et al., 2013; Montel et al., 2014), but the absence of *Lactobacillus* isolates from both winter RMs of native Epirus breeds (Table 3) was an unexpected finding. A similar rare occasion was a Brazilian raw milk type produced by Lacaune, Santa Ines and crossbred sheep breeds in which enterococci accounted for the total LAB isolates (*E. faecium,* 56.25 %; *E. durans*, 31.25 %; *E. casseliflavus*, 12.5 %) whereas no lactobacilli were found (Acurcio et al., 2014). In this study, the low (<4 log CFU/mL) prevalence of indigenous lactobacilli probably was an ecological characteristic of the fresh RM1 and RM2 samples depending on natural contamination during and after milking rather than it was due to a high in situ antagonistic activity of enterococci which also were subdominant (<4.2 log CFU/mL) of the dominant mesophilic LAB on the MRS/30 °C plates (i.e., MRS agar is highly selective for mesophilic *Lactobacillus* spp. when they prevail naturally).

3.3. Polyphasic identification of the raw sheep milk isolates at the species/subspecies

All ten *Leuconostoc* isolates (Group A) were perfectly identified (100 % 16S rRNA gene sequence similarity) with eight different strains of *Leuconostoc mesenteroides* (Table 4). Next, all of them and the three reference strains ACA-DC 0231, ACA-DC 0493 and ACA-DC 0750 were confirmed to belong to *Ln. mesenteroides* since all possessed the *rpoB* gene (Fig. 2), which is specific for this species (Ricciardi et al., 2020). The multiplex-PCR method further distinguished them in two clusters: one major cluster A comprising eight RM isolates and ACA-DC 0231 which possessed *dsr* and *sorA,* but lacked *araA,* and one minor cluster B comprising two RM1 isolates only, KFM3 and KFM9, which possessed *araA* and *dsr,* but lacked *sorA* (Fig. 2). Of special note, none of the remaining two dairy reference strains clustered with KFM3 and KFM9; instead, ACA-DC 0493 possessed *dsr* only whereas ACA-DC 0750 possessed all three genes above (Fig. 2). According to the decision tree of

Table 3
Phenotypic characterization and basic grouping of 42 coccoid LAB isolates from the two 'antilisterial' raw milk batches (RM1; RM2), and their numerical distribution in each raw milk batch and in association with the selectivity of the six LAB-growth-supportive enumeration/isolation agar media

LAB group/LAB genus	Basic differentiating characteristics								Raw milk batch		Total isolates	Enumeration/isolation agar medium					
	MA	CO_2	NH_3	10°C	45°C	4.0 %	6.5 %	KAA	RM1	RM2		MPCA 37 °C	MRS 30°C	MRS 45°C	M17 22°C	M17 42°C	KAA 37°C
Group A: *Leuconostoc*-like bacteria	CB	+	-	+	-	+	+	-	5	5	10	3	7				
Group B: *Enterococcus*	C	-	++	+	+	+	+	++	10	9	19		1	8			10
Group C: Mesophilic, arginine-positive *Lactococcus/ Streptococcus*	C	-	+	+	-	+	3/7	-	0	7	7	4			2	1	
Group D: Thermophilic *Streptococcus*	C	-	-	-	+	2/4	-	3/4	2	2	4	1		2		1	
Group E: Atypical coccoid homofermentative LAB	C	-	1/2	+	1/2	+	+	+/++	1	1	2		2				
Total LAB isolates									18	24	42	8	10	10	2	2	10

MA, Microscopic appearance as cocci (C) or coccobacilli (CB); CO_2, gas production from glucose; NH_3, ammonia production from arginine; 10 °C/45 °C, growth at 10 °C or 45°C; 4.0 %/6.5 %, growth in MRS or M17 broth with 4 % or 6.5 % sodium chloride; KAA, growth on kanamycin aesculin azide agar
+, positive reaction; -, negative reaction; ++, strong positive reaction; 2/4, 2 out of the 4 isolates in the group were positive.

Ricciardi et al. (2020), distinct gene profiles are possessed by the type strains of the *Ln. mesenteroides* subspecies, *mesenteroides* (*araA/sorA/dsr*), *jonggajibkimchii* (*araA/dsr*), and *dextranicum* (*dsr*), while the fourth subspecies *cremoris* lacks all three genes. On this basis, the subspecies identification of the strains ACA-DC 0750 as *Ln. mesenteroides* subsp. *mesenteroides* and ACA-DC 0493 as *Ln. mesenteroides* subsp. *dextranicum* was confirmed; whereas ACA-DC 0231, received as *Ln. mesenteroides* subsp. *dextranicum*, was found to be an atypical strain of the subspecies *dextranicum* that further possesses *sorA* (Fig. 2). Accordingly, all eight RM isolates in cluster A may be atypical strains of the subspecies *dextranicum* or, otherwise, atypical strains of the subspecies *mesenteroides* strains lacking *araA*. However, the most important finding was that the two distinct RM1 isolates in cluster B, KFM3 and KFM9, were assigned to *Ln. mesenteroides* subsp. *jonggajibkimchii,* a novel LAB isolated from kimchii; it was first described in 2017 (Jeon et al., 2017) and so far has been of limited association with milk and cheese products (Sanchez-Juanes et al., 2020). Recently, Rai and Tamang (2022) reported the isolation, 16S rRNA identification and probiotic characterization of some *Ln. mesenteroides* subsp. *jonggajibkimchii* strains from naturally fermented cow-milk and yak-milk products of Sikkim, India, along with numerous additional *Ln. mesenteroides* strains. Interestingly, the new subspecies was predominantly isolated from a specific fermented product only, hard-variety yak-milk Chhupri, while it was sporadic in soft-variety yak-milk and cow-milk Chhupri and undetectable in the other products, dahi, mohi, and philu (Rai and Tamang, 2022). The other three *Ln. mesenteroides* subspecies, *mesenteroides, dextranimum* and *cremoris,* occur commonly in raw milk and traditional cheeses, most often at subdominant levels to other LAB (Hemme and Foucaud--Scheunemann, 2004; Quigley et al., 2013; Ruppitsch et al., 2021). Generally, they metabolize lactose and citrate and thereby are beneficial, gas- and flavor-forming non-starter LAB (NSLAB), often included in natural undefined mesophilic starters (Frantzen et al., 2017), while *Ln. mesenteroides* subsp. *cremoris* strains are industrial dairy starters. *Ln. mesenteroides* is the primary *Leuconostoc* species in most traditional Greek cheeses, especially in those made of raw milk (Litopoulou-Tzanetaki and Tzanetakis, 2014; Gantzias et al., 2020; Zoumpopoulou et al., 2020). Regarding its prevalence in Greek raw sheep milk, limited data exist. Samelis et al. (2009) found only three *Ln. mesenteroides* subsp. *mesenteroides* among 49 LAB isolates from two bulk RM batches and Tsigkrimani et al. (2022a) reported the subdominant presence of *Ln. mesenteroides* in two out of five bulk RM batches intended for cheese making.

All 19 *Enterococcus* isolates (Group B) were first identified at the species by the IGS method (Fig. 3A), using *E. faecium* KE82, *E. durans* KE100, and *E. faecalis* GL322 as reference strains (Vandera et al., 2020). According to their profiles, they were assigned to *E. faecium* (6 isolates; Fig. 3A/3B), *E. faecalis* (3 isolates; Fig. 3B), and *E. durans* (8 isolates; Fig. 3A/3B, and Fig. S1), whereas two *Enterococcus* isolates from RM2, KFM49 and KFM56, gave IGS profiles that did not match with the reference strain of the above three species (Fig. 3B). Therefore, KFM49 and KFM56, along with *E. faecium* KFM17, KFM28, KFM29 and *E. durans* KFM6 selected for their m-Ent+ antilisterial activity (viz. Section 3.4), were subjected to 16S rRNA gene sequencing to elucidate or confirm their species identification, respectively. Results (Table 4) revealed that (i) strain KFM49 also belongs to *E. durans*; (ii) strain KFM56 was confirmed to be distinct, identified as *Enterococcus hirae*; (iii) strain KMF6 was confirmed to be *E. durans*; and (iv) strains KFM17, KFM28 and KFM29 were confirmed to belong to *E. faecium* (Table 4). The varying prevalence rates and multiple controversial roles of enterococci, particularly *E. faecalis, E. faecium*, and *E. durans*, in raw milk and cheese (Giraffa, 2003; Quigley et al., 2013; Dapkevicius et al., 2021), including Greek cheeses (Moreno et al., 2006; Litopoulou-Tzanetaki and Tzanetakis, 2014; Vandera et al., 2019), are well documented. Relative to this study, *E. faecalis* prevailed before thermization in two Epirus raw sheep/goat bulk milks followed by *E. faecium* and *E. durans* (Samelis et al., 2009), whereas *E. faecium* was more frequent than *E. faecalis* and *E. durans* was not detected in another five raw sheep bulk milks (Tsigkrimani et al., 2022a).

The species identification of the remaining 13 RM isolates was based on the 16S rRNA method only (Table 4). Specifically, all seven mesophilic, homofermentative coccoid LAB isolates from RM2 (Group C; Table 3) were perfectly identified (100 % homogeny) with five different strains of *Streptococcus parauberis;* all were similar to strains previously isolated in South Korea (Table 4). The prevalence of *S. parauberis* in RM2 should be regarded as a safety concern rather than as a beneficial trait because this species is amongst the minor streptococci involved in bovine mastitis, with its close relative *Streptococcus uberis* (Pitkala et al., 2008) and two other species, *Streptococcus agalactiae* and *S. dysgalactieae,* being the major ones (Quigley et al., 2013; Alnakip et al., 2020). Indeed, McDonald et al. (2005) used PCR-RFLP analysis of 16S-23S ribosomal DNA to reveal that 53 out of 100 streptococcal field isolates from cows with clinical and subclinical mastitis belonged primarily to *S. uberis* (47) and only six to *S. parauberis*. Similarly based on their RFLP patterns, Pitkala et al. (2008) reported that only two

Table 4
Molecular identification of representative raw sheep milk LAB isolates by 16S rRNA sequencing analysis[1]

Phenotypic Group in Table 4	Raw milk Batch	Isolate code	Genotypic species identification	Closest relative reference strain in BLAST	16S rRNA gene seq. similarity (%)
A	RM1	KFM2	*Leuconostoc mesenteroides*	MN229548.1	100
	RM1	KFM3	*Leuconostoc mesenteroides*	NR040818.1	100
	RM1	KFM7	*Leuconostoc mesenteroides*	MN229548.1	100
	RM1	KFM9	*Leuconostoc mesenteroides*	MN173332.1	100
	RM1	KFM10	*Leuconostoc mesenteroides*	JN853602.1	100
	RM2	KFM31	*Leuconostoc mesenteroides*	CP020731.1	100
	RM2	KFM36	*Leuconostoc mesenteroides*	MN994413.1	100
	RM2	KFM38	*Leuconostoc mesenteroides*	CP020731.1	100
	RM2	KFM39	*Leuconostoc mesenteroides*	CP065995.1	100
	RM2	KFM40	*Leuconostoc mesenteroides*	KT124572.1	100
B	RM1	KFM6	*Enterococcus durans*	LR607335.1	100
	RM1	KFM17	*Enterococcus faecium*	CP041261.3	100
	RM1	KFM28	*Enterococcus faecium*	CP041261.3	100
	RM1	KFM29	*Enterococcus faecium*	LR135782.1	100
	RM2	KFM49	*Enterococcus durans*	KF147885.1	100
	RM2	KFM56	*Enterococcus hirae*	MK533782.1	100
C	RM2	KFM32	*Streptococcus parauberis*	MT579801.1	100
	RM2	KFM33	*Streptococcus parauberis*	MT597919.1	100
	RM2	KFM34	*Streptococcus parauberis*	CP025420.1	100
	RM2	KFM35	*Streptococcus parauberis*	CP025420.1	100
	RM2	KFM41	*Streptococcus parauberis*	MN758826.1	100
	RM2	KFM42	*Streptococcus parauberis*	CP025420.1	100
	RM2	KFM54	*Streptococcus parauberis*	MT579786.1	100
D	RM1	KFM5	*Streptococcus equinus*	MF429207.1	100
	RM1	KFM26	*Streptococcus gallolyticus*	CP050959.1	99.93
	RM2	KFM55	*Streptococcus lutetiensis*	LS483348.1	100
	RM2	KFM60	*Streptococcus lutetiensis*	LS483403.1	100
E/Atypical	RM1	KFM8	*Lactococcus lactis*	MW882987.1	100
	RM2	KFM37	*Enterococcus hermaniensis*	MF423827.1	99.93

[1] Additional *Enterococcus* spp. isolates from RM1 and RM2 were identified at the species based on their IGS profile similarity with reference/control strains, according to the results shown in Fig. 2 and in Supplementary Fig. 1.

subclinical *S. parauberis* isolates occurred within 137 clinical and subclinical isolates of the *S. uberis/parauberis* group, concluding that the former species is not a frequent cause of bovine intra-mammary infections in Finland. More recent studies combined RFLP-PCR with MALDI-TOF MS to gain a better discrimination between all major and minor streptococci involved in mastitis (Alnakip et al., 2020; Rosa et al., 2022). Specifically, Rosa et al. (2022) focused on the identification of 57 streptococci from the raw milk of sheep and goats with mastitis and found that the most prevalent species was *S. uberis* (89.5 %) followed by *S. parauberis* (3.5 %), *S. dysgalactiae* (3.5 %) and *Streptococcus gallolyticus* (1.8 %).

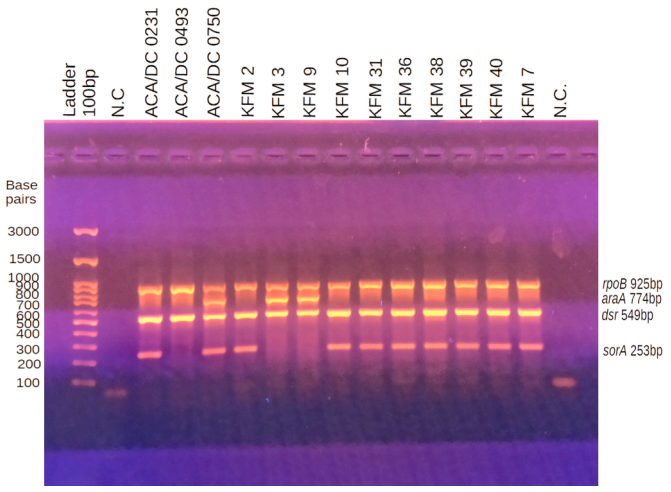

Fig. 2. Multiplex PCR profiling of ten *Leuconostoc mesenteroides* raw sheep milk (KFM) isolates. Lane 1: Nippon Genetics ready-to-use DNA ladder, 100 to 3000 bp fragments; Lane 2: Negative control; Lane 3: *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0231; Lane 4: *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0493; Lane 5: *Ln. mesenteroides* subsp. *mesenteroides* ACA-DC 0750; Lanes 6 – 15: *Ln. mesenteroides* KFM isolates; Lane 16: negative control. At 925 bp is the *rpoB* gene band; at 774 bp is the *araA* gene band; at 549 bp is the *dsr* gene band; at 253 bp is the *sorA* gene band.

The four thermophilic RM isolates (Group D; Table 3) were confirmed to be diverse *Streptococcus* spp. (Table 4). The two RM1 isolates, KFM5 and KFM26, were identified at 100 % and 99.93 % 16S rRNA homogeny with *Streptococcus equinus* and *S. gallolyticus*, respectively; whereas the two RM2 isolates, KFM55 and KFM60, shared a 100 % homogeny with two *Streptococcus lutetiensis* strains from UK (Table 4). *S. equinus* and *S. gallolyticus* are also considered minor streptococci involved in bovine mastitis along with *S. parauberis, S. salivarius* and *S. canis* (Alnakip et al., 2020), while *S. lutetiensis,* previously termed *Streptococcus bovis* type II/1, possesses the general characteristics of a contagious mastitis pathogen although has rarely been associated with bovine mastitis (Chen et al., 2021). Taxonomically, all three thermophilic RM species identified herein, *S. equinus, S. gallolyticus* and *S. lutetiensis,* are members of the *S. bovis/S. equinus* complex, which has been subjected to several reclassifications after 2000 (Poyart et al., 2002; Schlegel et al., 2003); it is a rather controversial group of streptococci, like *Enterococcus*, because it includes pathogenic but also desirable species/subspecies that are well-adapted to dairy niches (Papadimitriou et al., 2014). Actually, *S. gallolyticus* includes the mastitis pathogen subspecies *gallolyticus* (formerly *S. gallolyticus*) and the blood-associated subspecies *pasteurianus* (formerly *S. pasteurianus*), both implicated in human endocarditis, as well as, the beneficial dairy-specific subspecies *macedonicus* (formerly *S. macedonicus*) first isolated from traditional Greek Kasseri cheese (Schlegel et al., 2003; Papadimitriou et al., 2014). Whereas *S. lutetiensis* previously was the subspecies *coli* of another species in the same complex, *Streptococcus infantarius* (Poyart et al., 2002); *S. infantarius* subsp. *infantarius* remains valid. Several recent studies, particularly dealing with soft acid-curd artisan Turkish cheeses, have linked the prevalence of *S. lutetiensis, S. gallolyticus, S. macedonicus* and *S. infantarius* subsp. *infantarius* with beneficial biotechnological and antagonistic (bacteriocin) properties and/or gene clusters in their genome (Papadimitriou et al., 2014; Hill et al., 2020; Demirci et al., 2021; Özkan et al., 2021b; Tsuda and Kodama, 2021; Aktas et al., 2022; Güley et al., 2022; de Oliveira et al.,

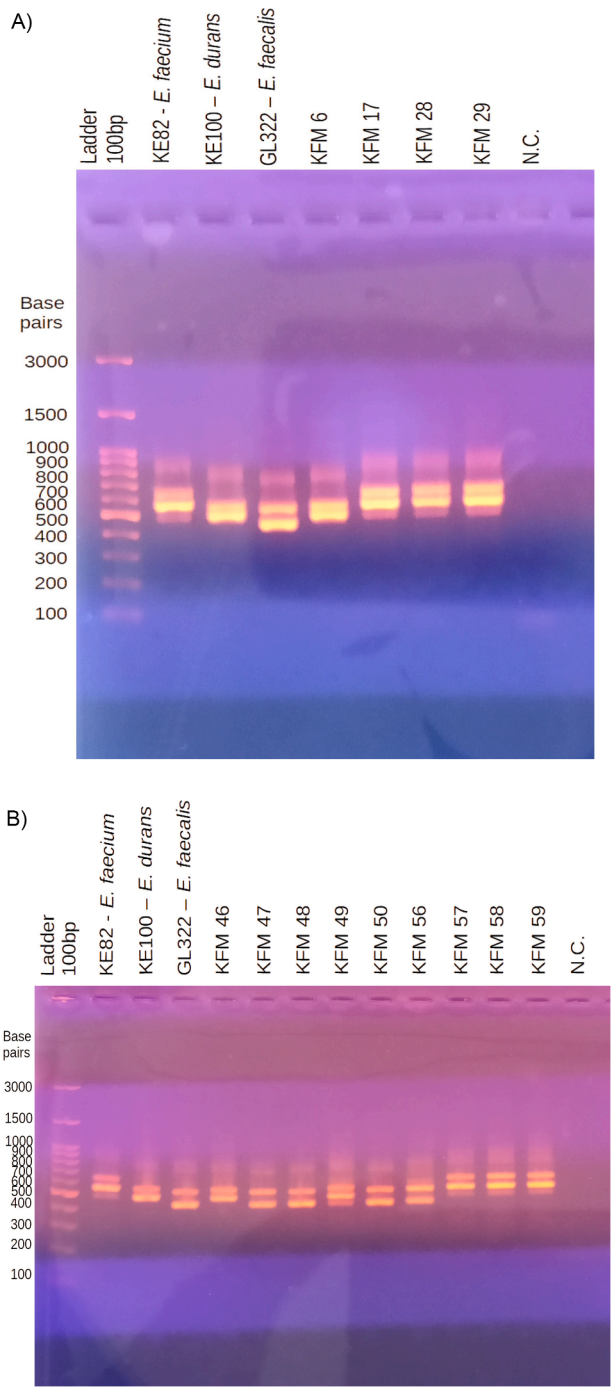

Fig. 3. Species identification of raw sheep *Enterococcus* spp. KFM isolates using the IGS method. A) Lane 1: Nippon Genetics ready-to-use DNA ladder, 100 to 3000 bp fragments; Lane 2*: E. faecium* KE82; Lane 3: *E. durans* KE100; Lane 4: *E. faecalis* GL322; Lane 5: KFM6; Lane 6: KFM17; Lane 7: KFM28; Lane 8: KFM29; Lane 9: Negative control B) Lane 1: Nippon Genetics ready-to-use DNA ladder, 100 to 3000 bp fragments; Lane 2*: E. faecium* KE82; Lane 3: *E. durans* KE100; Lane 4: *E. faecalis* GL322; Lanes 5 - 9: KFM46 – KFM50; Lanes 10 - 13: KFM56 – KFM59; Lane 14: negative control.

2022). The above phylogenetic and biotechnological cheese ecology studies are emphasized because (i) the isolate *S. gallolyticus* KFM26 (Table 4) was alternatively identified at 99.93 % similarity of its 16S rRNA gene sequence with *S. pasteurianus* (a Dutch strain with MK330581.1), suggesting that KFM26 is a *S. gallolyticus* subsp. *pasteurianus* strain; (ii) the isolate KFM55 was clearly identifiable as *S. lutetiensis*, but the other diverse *S. lutetiensis* isolate KFM60 from RM2 was alternatively identified as *S. infantarius* (100 % homogeny with another Dutch strain MK330572.1), and (iii) both KFM55 and KFM60 strains showed a strong direct antagonistic antilisterial activity, as it will be addressed below (viz. Section 3.4). Therefore, additional genotypic studies with species/subspecies specific gene primers are required for assuring the identification of the four thermophilic *Streptococcus* isolates from RM.

Finally, the two atypical coccoid LAB isolates (Group E; Table 3) were confirmed to belong to two distinct LAB species (Table 4). KFM8 was identified as *Lactococcus lactis,* whereas the 16S rRNA gene identification of KFM37 was doubtful, i.e., *E. hermanniensis/E. devriesei/E. pallens*, sharing the highest similarity at 99.93 % with *E. hermanniensis* (Table 4). The latter species was originally isolated from modified-atmosphere packaged broiler meat, belongs to the *Enterococcus avium* group and, indeed, its closer phylogenetic neighbor is *E. pallens* (Koort et al., 2004). Of note, a recent study found *E. hermanniensis* (1.6 %) along with *E. durans* (4.8 %) to be subdominant of *E. faecium* (58.7 %) and *E. faecalis* (31.8 %) in Brazilian artisanal cheeses (Margalho et al. 2020).

3.4. Direct antilisterial activity, bacteriocin-mediated activity, and bacteriocin encoding genes of the raw sheep milk LAB isolates

Three *E. faecium* isolates, KFM17, KFM28, KFM29, showed strong direct in vitro antagonistic activity against *L. monocytogenes* no.10 in the M17 agar overlay assays, while another three *E. faecium* isolates, KFM57, KFM58, KFM59, and *E. durans* KFM6, showed moderate activity (Table 5). Of note, all strongly active *E. faecium* isolates and the most active *E. durans* KFM6 isolate were recovered from RM1, whereas only *Enterococcus* isolates of moderate to weak activity were found in RM2, in accordance with the Table 2 results. No other LAB isolate showed a strong antilisterial activity in the M17 agar overlays, except of the two *S. lutetiensis* KFM55 and KFM60 strains recovered from RM2 (Table 5).

Eventually, only the CFS of *E. faecium* KFM17, KFM28 and KFM29 following growth for 24 h at 30 °C in MRS or M17 broth retained a strong enterocin-mediated antilisterial activity, even after the CFS pH was neutralized; however, this activity was lost after treatment of the CFS of the above *E. faecium* isolates with proteinase K, α-chymotrypsin, and trypsin (Table 5), confirming the presence of enterocin/s. Based on similar previous findings critically discussed by Vandera et al. (2020), all antagonistic *Enterococcus* isolates in the M17 agar overlay assays were screened by PCR to detect active, but also silent or poorly expressed, enterocin-encoding genes in their genome. Consistent with their strong antilisterial activity in vitro, the *E. faecium* KFM17, KFM28 and KFM29 isolates possessed the enterocin A-B-P genes, similarly to the reference strain *E. faecium* KE82 (Table 5); this was confirmed by sequencing of the *entA* (JF944896.1; 98.5 %), *entB* (HQ407492.1; 100 %) and *entP* (LN999988.1; 96 %) gel bands of KFM28. Consistent with its moderate activity, *E. durans* KFM6 possessed the *entA* and *entP* genes (Table 5). The *entA, entB* and *entP* bands detected by PCR in the above four most active RM isolates are shown in Fig. S2. Another three *E. faecium* isolates (KFM57, KFM58 and KFM59) and *E. hirae* KFM56 possessed *entA* only, whereas another two *E. durans* isolates (KFM46 and KFM49) possessed *entP* only. None of the remaining enterocin (L50A, L50B, AS-48, Bac31) or cytolycin genes were detected in any of the *Enterococcus* isolates from RM. Particularly the $cytL_L$ encoding cytolysin, a complex class I bacteriocin possessed by clinical *E. faecalis* strains (Cox et al., 2005), was detected only in the β-hemolytic and virulent *E. faecalis* GL320 and ATCC 29212^{TM} (Table 5). This clarification is required because Vandera et al. (2020) reported that the non-virulent *E. faecalis* GL322 and, most uncommonly, *E. durans* KE108 possessed $cytL_L$; those previous findings were not confirmed in the present study. Instead, the m-Ent+ *E. durans* KE108 was found to possess *entA* additionally to *entP* and *bac31* (Table 5).

The frequent occurrence of m-Ent+ *E. faecium* strains in raw milk and traditional cheeses made from raw or thermized milk has become a very common finding in recent years (Ghairi et al., 2008; Edalatian et al.,

Table 5
Antilisterial activity of raw sheep milk LAB isolates detected by the simple agar overlay technique (direct activity), and confirmation of the presence of active enterocin molecules in the *Enterococcus* spp. cell-free supernatants (CFS) in association with the PCR detection of enterocin genes in their genome.

LAB strain	M17 agar overlay [1]	CFS/Well assay [2]	Enzyme-treated CFS [3]	Enterocin gene detected by PCR [4]							
				entA	*entB*	*entP*	*entL50A*	*entL50B*	*entAs-48*	*bac31*	*CytL*$_L$
Control strains											
E. faecium KE82	++	++	-	+	+	+	-	-	-	-	-
E. durans KE100	+	-	NT	-	-	+	-	-	-	-	-
E. durans KE108	+	-	NT	+	-	+	-	-	-	+	-
E. faecium 315 VR	++	NT	NT	+	-	+	-	-	-	-	-
E. faecalis ATCC 29212^{TM}	++	-	NT	-	-	-	-	-	-	-	+
E. faecalis GL320	++	-	NT	-	-	-	-	-	-	-	+
Raw milk isolates											
E. faecium KFM17	++	++	-	+	+	+	-	-	-	-	-
E. faecium KFM28	++	++	-	+	+	+	-	-	-	-	-
E. faecium KFM29	++	++	-	+	+	+	-	-	-	-	-
E. faecium KFM57	+	-	NT	+	-	-	-	-	-	-	-
E. faecium KFM58	+	-	NT	+	-	-	-	-	-	-	-
E. faecium KFM59	+	-	NT	+	-	-	-	-	-	-	-
E. durans KFM6	+	-	NT	+	-	+	-	-	-	-	-
E. durans KFM46	(+)	-	NT	-	-	+	-	-	-	-	-
E. durans KFM49	(+)	-	NT	-	-	+	-	-	-	-	-
E. hirae KFM56	-	-	NT	+	-	-	-	-	-	-	-
S. lutetiensis KFM55	++	-	NT	NA	NA	NA	NA	NA	NA	NA	NA
S. lutetiensis KFM60	++	-	NT	NA	NA	NA	NA	NA	NA	NA	NA

[1] ++, strong clearness/growth inhibition of *L. monocytogenes*; +, moderate clearness; (+) faint clearness zone; -, no clearness around the streaked colonies
[2] ++, large inhibition zone (> 5–15 mm depending on the CFS (MRS or M17) medium; -, no inhibition zone
[3] The active neutralized CFS were confirmed to loose antilisterial activity after treatment with 1 mg/mL of proteinase K, or α-chymotrypsin, or trypsin.
[4] +, presence of the enterocin gene tested; -, absence of the enterocin gene tested.
NT, not tested; NA, not applicable as regards enterocin gene detection in the two *Streptococcus lutetiensis* isolates.

2012; Nami et al., 2019; Vandera et al., 2020; Garmasheva and Oleschenko, 2023), particularly as regards strains profiling the *entA/entB* or *entA/entB/entP* genes and originating from Italian goat milk (Cocolin et al., 2007), or Greek raw sheep milk (Chanos and Williams, 2011) or thermized sheep/goats' bulk milks (Vandera et al., 2018). Although enterocin production is strain-specific and dependent on the culture conditions, it is generally accepted that *E. faecium* A-B-P or A-B strains manifest a stronger antilisterial activity than *E. faecium* strains processing *entA* only, or *entA* and *entP*, or *entB* and *entP* (Cocolin et al., 2007; Nami et al., 2019; Vandera et al., 2020; Garmasheva and Oleschenko, 2023), mainly because enterocins A and B act synergistically (Table 5). *E. faecium* strains processing the *entA/entB/entL50A/50B* or *entA/entB/entL50A/entQ* profiles were reported to occur in raw sheep milk in northern Greece (Chanos and Williams, 2011) and artisanal Iranian dairy products (Nami et al., 2019), respectively. Most of them, however, show low antilisterial activity. We have also detected *E. faecium* KE77, a strange Graviera cheese isolate that possesses four enterocin genes (A, B, P, 31) but otherwise is of low to negligible activity against *L. monocytogenes* in synthetic broth and milk cocultures (Vandera et al., 2020). Such findings may indicate horizontal transfer of enterocin encoding genes between *Enterococcus* strains isolated from the same niche; some or even all transferred genes may remain silent or are poorly expressed, as also the results in Table 5 suggest. Notably, active *E. faecalis* strains possessing the species-specific enterocin AS-48 gene haven't been isolated from Greek sheep/goat milks so far (Samelis et al., 2009; Chanos et al., 2011; Vandera et al., 2018, 2020), but they were common in sheep, goat or cow milks from Spain (Rodriguez et al., 2000). Finally, the direct antilisterial activity of the *S. lutetiensis* KFM55 and KFM60 strains might be due to bacteriocin/s; however, their inactive CFS against *L. monocytogenes* no.10 (Table 5) suggests that the active bacteriocin molecule/s may remain 'bound' on the producer strain's cell surface; this was previously noted for the β-hemolytic, cytolysin-positive *E. faecalis* GL311/GL320 strain genotype, which otherwise was strongly active against *L. monocytogenes* in culture broth and sterile-raw milk co-cultures (Vandera et al., 2020).

3.5. Hemolytic activity, vancomycin resistance, and virulence genes of the raw milk isolates

All RM isolates were screened for hemolytic activity and possession of the *vanA, vanB, agg, ace, espA, IS16, hyl* and *gelE* genes. The main results are summarized in Table 6. No RM isolate was β-hemolytic. However, all *Enterococcus* and *Streptococcus* isolates showed α-hemolysis, including the selected isolates listed in Table 6, whereas all *Ln. mesenteroides* isolates and *Lc. lactis* KFM8 showed γ-hemolysis (data not tabulated). Three *E. faecalis* RM2 isolates possessed the *gelE* and/or *ace* virulence genes, singly (KFM48, KFM50) or combined (KFM47) (Table 6; the gels with the *gelE* and/or *ace* gene bands detected in the above *E. faecalis* isolates are shown in Fig. S3). All RM isolates lacked *vanA* and *vanB* for vancomycin resistance, and *agg, espA, hyl* and *IS16* virulence genes (data not tabulated), including the four, most active antilisterial, m-Ent+ strains of the *E. faecium/durans* group listed in Table 6. A strong β-hemolysis reaction was reconfirmed for the positive control strains, as well as, the *ace* and *gelE* possession by *E. faecalis* ATCC 29212^{TM} and the *vanA* presence in *E. faecium* 315VR. Of note, we also found that (i) *E. faecalis* ATCC 29212^{TM} possesses the *agg* and *espA* additionally to the *ace* and *gelE* genes; (ii) the β-hemolytic and *CytL*$_L$+ *E. faecalis* GL320 originally isolated from a fresh Galotyri PDO cheese is indeed a virulent strain sharing the profile *agg/ace/espA/gelE* with the ATCC strain; and (iii) *E. faecium* 315VR is also a virulent (*agg/espA/IS16/gelE* profiling) strain (Table 6).

The absence of *cytL*$_L$ gene (Table 5), the lack of β-hemolytic activity, and the absence of all six common virulence genes and both vancomycin resistance genes from the genome of all RM isolates of the *E. faecium/durans* group, including the four most active m-Ent+ strains in Table 6, are encouraging findings regarding their safe use as adjuncts in traditional Greek (Kefalotyri) cheese processing. Similar results were obtained for 17 Ent+ or m-Ent+ *E. faecium* strains from raw sheep milk in northern Greece (Chanos and Williams, 2011) and nine autochthonous Ent+ or m-Ent+ strains of the *E. faecium/durans* group from artisan Graviera and Galotyri PDO cheeses (Tsanasidou et al., 2021). Generally, *E. faecium* strains that possess hemolysin/cytolysin, virulence and/or vancomycin-resistance genes do not seem widespread in raw milk and

Table 6
Hemolytic activity, and presence of vancomycin resistance and virulence genes in *Enterococcus faecalis* and the selected multiple enterocin-producing (m-Ent+) strains of the *E. faecium/durans* group isolated from raw sheep milk

Enterococcus strain	Hemolytic activity	Vancomycin resistance genes		Virulence genes					
		vanA	*vanB*	*agg*	*ace*	*espA*	*hyl*	*IS16*	*gelE*
Control positive virulent strains									
E. faecalis ATCC 29212^{TM}	β	-	-	+	+	+	-	-	+
E. faecalis GL320	β	-	-	+	+	+	-	-	+
E. faecium 315 VR	β	+	-	+	-	+	-	+	+
Control negative (safe) dairy strains									
E. faecium KE82 (m-Ent+)	α	-	-	-	-	-	-	-	-
E. durans KE100 (entP+)	α/γ	-	-	-	-	-	-	-	-
E. durans KE108 (m-Ent+)	α	-	-	-	-	-	-	-	-
Raw milk isolates									
E. faecalis KFM47	α	-	-	-	+	-	-	-	+
E. faecalis KFM48	α	-	-	-	-	-	-	-	+
E. faecalis KFM50	α	-	-	-	+	-	-	-	-
E. faecium KFM17 (m-Ent+)	α	-	-	-	-	-	-	-	-
E. faecium KFM28 (m-Ent+)	α	-	-	-	-	-	-	-	-
E. faecium KFM29 (m-Ent+)	α	-	-	-	-	-	-	-	-
E. durans KFM6 (m-Ent+)	α	-	-	-	-	-	-	-	-

+, presence of the gene tested; -, absence of the gene tested.
Hemolysis type: β-hemolysis (clearness of the red color around the streaked colony growth on 5 % sheep blood agar); α-hemolysis (greening of the red color around the streaked colony growth on 5 % sheep blood agar); γ-hemolysis (no red color change around the streaked colony growth on 5 % sheep blood agar).

traditional cheeses in Greece and, in recent years, few unsafe *E. faecium* strains were retrieved from various cheeses of neighboring countries (Hammad et al., 2015; Özkan et al., 2021a). The incidence of virulence factors in food isolates of *E. faecium* is strain-specific, like *E. faecium* ST7319ea (Fugaban et al., 2021). In general, few strains within an isolated group, most of which belonged to *E. faecalis*, harbored the *ace* and *gelE* genes, as the *E. faecalis* KFM47, KFM48 and KFM50 strains (Table 6), or the agg, *IS16, espA* and *hyl*-like genes (Morandi et al., 2015; Hammad et al., 2015; Gaglio et al., 2016; Ruiz et al., 2016; Domingos-Lopez et al., 2017). Particularly for *E. faecium*, the absence of the *IS16, espA* and *hyl*-like virulence gene markers associated with clinical strains of the species (confirmed in *E. faecium* 315VR) (Table 6), should be a prerequisite for strains to be considered safe for use in food. Although *E. durans* has been less investigated than *E. faecium*, safe *E. durans* strains of dairy origin have also been selected (Terzić-Vidojević et al., 2015; Laucová et al., 2021).

The absence of the *gelE, hyl* or cytolysin genes from all thermophilic *Streptococcus* RM isolates, particularly *S. gallolyticus* KFM5, *S. lutetiensis* KFM55 and *S. lutetiensis/infantarius* KFM60, was another positive finding, consistent with the findings reported by Özkan et al. (2021b) regarding beneficial properties of *S. gallolyticus, S. lutetiensis* and *S. infantarius* cheese isolates. The variable virulence gene profiles of field strains belonging to the primary two *Streptococcus* groups, *S. uberis/parauberis* (Zhang et al., 2020) and *S. bovis/equinus* (Özkan et al., 2021b) associated with mastitis in cows and small ruminants, is an important safety concern. Additional virulence STR genes (*asa1, mrp, sly, bay, bca, speG, scpB, ssa*) were tested for the *S. bovis/equinus* complex and *S. lutetiensis* in particular (Chen et al., 2021; Özkan et al., 2021b); whereas the virulence genes (*sua, pauA, skc, gapC, hasC*) of the *S. uberis/parauberis* group (Zhang et al., 2020) differ from the *Enterococcus*-specific virulence genes tested in this study. To our knowledge, no previous culture-dependent studies have reported the presence/prevalence of *S. parauberis* isolates in Greek raw milk or traditional cheese; however, a recent targeted (16S rRNA) metagenomic study reported the prevalence of *S. parauberis* (OTU 50) in the microbiota of artisanal Gidotyri cheese (Nelli et al., 2023). Therefore, further studies are needed to assess the biochemical characteristics and virulence potential, as well as the antibiotic resistance, of the present *S. parauberis* isolates from RM.

3.6. Selection of safe raw sheep milk LAB strains for use as bioprotective adjunct cultures in traditional Kefalotyri cheese production – practical technological aspects

Altogether the results of this study confirm that raw sheep milk from native breeds crossbred in Epirus, Greece, remains a rich pool of indigenous, probably unexplored, LAB strains with desirable antagonistic properties. Actually, the two 'antilisterial' RM batches were bulk-tank milks intended for Kefalotyri production, a Greek hard cheese traditionally processed from raw sheep milk with increased salt (>3.5-6 %) levels, thereby harboring salt-tolerant enterococci, mainly *E. faecium* and *E. faecalis*, at high predominant levels (Litopoulou-Tzanetaki, 1990). We showed (Tables 2–6) that the most prevalent LAB in RM1 and RM2 were *Ln. mesenteroides* and safe m-Ent+ strains of the *E. faecium/durans* group, both having profound growth ability at 6.5 % salt. However, in general, beneficial *Leuconostoc* and *Enterococcus* present in raw milk do not necessarily reach sufficiently high population levels in the resultant cheeses because either they grow slowly in milk or they are poor acidifiers outcompeted by more aciduric LAB (*Lc. lactis, S. thermophilus*, lactobacilli) during traditional (Greek) raw milk cheese fermentations. Furthermore, because traditional Kefalotyri cheese is nowadays processed after pasteurization or thermization (63–65 °C for 30–60 s) of the raw sheep milk, many beneficial LAB contaminants present in Epirus RM would be inactivated along with the vast majority of undesirable non-LAB spoilage or pathogenic bacteria. This is particularly true for *Ln. mesenteroides* and wild antagonistic lactococci in RM, as it happened to the prevalent *Lc. lactis* (20.4 % of isolated LAB) strains, including the indigenous novel NisA+ *Lc. lactis* subsp. *cremoris* M78/M104 strain genotype (Parapouli et al., 2013) in bulk raw Epirus sheep/goats' milks postthermization (Samelis et al., 2009). Wild *Lc. lactis* strains, several of which produce nisin A, nisin Z, and other antilisterial bacteriocins, occur commonly in raw milk (Rodriguez et al., 2000; Perin et al., 2012; Quigley et al., 2013). Surprisingly, in this study, only one *Lc. lactis* KFM8 that did not appear to be a Bac+ strain was isolated from RM1; most surprisingly, no lactobacilli were isolated from RM1 and RM2 (Table 4), although *Lactiplantibacillus plantarum* was amongst the most frequently isolated species from bulk sheep or sheep/goat milks (Samelis et al., 2009; Tsigkrimani et al., 2022a). These findings confirm that the microbial (LAB) ecology of raw milk may be very diverse between countries, regions, seasons, or even farms and dairy plants from year to year. Nevertheless, this study confirmed that safe, m-Ent+ strains of the *E. faecium/durans* group is the commonest and most promising

antagonistic (antilisterial) LAB fraction isolated from Greek raw or thermized bulk milks and traditional, naturally fermented Greek raw or thermized milk cheeses (Chanos and Williams, 2011; Vandera et al., 2018, 2019; Tsanasidou et al., 2021; Tsigkrimani et al., 2022a), but the commercial application of such strains already validated in pilot-plant cheeses, like our enterocin A-B-P-producer *E. faecium* KE82, is still hampered by legislation (Dapkevicious et al., 2021). Safe autochthonous enterococci with desirable biotechnological, bioprotective (antilisterial) and probiotic properties are also inactivated partially by thermization, and almost entirely, by pasteurization or boiling of raw milk. In conclusion, from the present RM LAB isolates, the two prevalent *Ln. mesenteroides* clusters, particularly the two *Ln. mesenteroides* subsp. *jonggajibkimchii* KFM3 and KFM9 strains in cluster B, the three safe antilisterial strains of the *E. faecium/durans* group (KFM17/KFM28, KFM29 and KFM6), and the only wild *L. lactis* KFM8 strain should be validated further as potential costarter or adjunct strains in traditional Kefalotyri cheese making trials, in replacement of counterpart LAB strains that would probably be inactivated by thermization. Additional in-depth studies are required to evaluate benefits versus risks from applying selected wild thermophilic streptococci as adjuncts in Kefalotyri cheese, especially the two antagonistic *S. lutetiensis* strains KFM55 and KFM60. On the other hand, the prevalence of α-hemolytic pyogenic streptococci in Epirus raw milk, mainly *S. parauberis* in RM2, requires further consideration in respect to measures linked with subclinical mastitis cases in the mixed Karamaniko/Karagouniko sheep breeds in each yard and, overall, the farm milk hygiene.

CRediT authorship contribution statement

Eleni Sioziou: Methodology, Formal analysis, Investigation, Data curation, Writing – original draft. **Athanasia Kakouri:** Formal analysis, Data curation. **Loulouda Bosnea:** Funding acquisition, Writing – review & editing. **John Samelis:** Conceptualization, Methodology, Formal analysis, Resources, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Supplementary materials

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References

Acurcio, L.B., Souza, M.R., Nunes, A.C., Oliveira, D.L.S., Sandes, S.H.C., Alvim, L.B., 2014. Isolation, enumeration, molecular identification and probiotic potential evaluation of lactic acid bacteria isolated from sheep milk. Arq. Bras. Med. Veterinária e Zootec. 66, 940–948.

Aktaş, M.H., Erdoğan, A., 2022. Characterization of technological properties of lactic acid bacteria isolated from Turkish Beyaz (white) cheese. J. Food Process. Preserv. 46, e16837.

Alexopoulos, A., Tzatzimakis, G., Bezirtzoglou, E., Plessas, S., Stavropoulou, E., Sinapis, E., Abas, Z., 2011. Microbiological quality and related factors of sheep milk produced in farms of NE Greece. Anaerobe 17, 276–279.

Alnakip, M.E.A., Rhouma, N.R., Abd-Elfatah, E.N., Quintela-Baluja, M., Böhme, K., Fernández-No, I., Bayoumi, M.A., Abdelhafez, M.M., Taboada-Rodriguez, A., Calo-Mata, P., 2020. Discrimination of major and minor streptococci incriminated in bovine mastitis by MALDI-TOF MS fingerprinting and 16S rRNA gene sequencing. Res. Vet. Sci. 132, 426–438.

Anastasiou, R., Kazou, M., Georgalaki, M., Aktypis, A., Zoumpopoulou, G., Tsakalidou, E., 2022. Omics approaches to assess flavor development in cheese. Foods 11, 188.

Angelidis, A.S., Komodromos, D., Giannakou, R., Arsenos, G., Gelasakis, A.I., Kyritsi, M., Filioussis, G., Hadjichristodoulou, C., Torounidou, P., Papa, A., Sergelidis, D., 2020. Isolation and characterization of *Staphylococcus aureus* and methicillin-resistant *Staphylococcus aureus* (MRSA) from milk of dairy goats under low-input farm management in Greece. Vet. Microbiol. 247, 108749.

Apostolakos, I., Paramithiotis, S., Mataragas, M., 2023. Comparative genomic analysis reveals the functional traits and safety status of lactic acid bacteria retrieved from artisanal cheeses and raw sheep milk. Foods 12, 599.

Chanos, P., Williams, D.R., 2011. Anti-*Listeria* bacteriocin-producing bacteria from raw ewe's milk in northern Greece. J. Appl. Microbiol. 110, 757–768.

Chen, P., Qiu, Y., Liu, G., Li, X., Cheng, J., Liu, K., Qu, W., Zhu, C., Kastelic, J.P., Han, B., 2021. Characterization of *Streptococcus lutetiensis* isolated from clinical mastitis of dairy cows. J. Dairy Sci. 104, 702–714.

Cocolin, L., Foschino, R., Comi, G., Fortina, M.G., 2007. Description of the bacteriocins produced by two strains of *Enterococcus faecium* isolated from Italian goat milk. Food Microbiol. 24, 752–758.

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, 2276.

Cox, C.R., Coburn, P.S., Gilmore, M.S., 2005. Enterococcal cytolysin: a novel two component peptide system that serves as a bacterial defense against eukaryotic and prokaryotic cells. Curr. Protein Pept. Sci. 6, 77–84.

Dapkevicius, M., de, L.E., Sgardioli, B., Câmara, S.P.A., Poeta, P., Malcata, F.X., 2021. Current trends of enterococci in dairy products: a comprehensive review of their multiple roles. Foods 10, 821.

de Oliveira, I.M.F., Godoy-Santos, F., Oyama, L.B., Moreira, S.M., Dias, R.G., Huws, S.A., Creevey, C.J., Mantovani, H.C., 2022. Whole-genome sequencing and comparative genomic analysis of antimicrobial producing *Streptococcus lutetiensis* from the rumen. Microorganisms 10, 551.

Demirci, T., Akin, N., Atik, D.S., Özkan, E.R., Dertli, E., Akyol, İ., 2021. Lactic acid bacteria diversity and dynamics during ripening of traditional Turkish goatskin Tulum cheese produced in Mut region assessed by culturing and PCR-DGGE. LWT 138, 110701.

Domingos-Lopes, M.F.P., Stanton, C., Ross, P.R., Dapkevicius, M.L.E., Silva, C.C.G., 2017. Genetic diversity, safety and technological characterization of lactic acid bacteria isolated from artisanal Pico cheese. Food Microbiol. 63, 178–190.

Doyle, C.J., Gleeson, D., Jordan, K., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P. D., 2015. Anaerobic sporeformers and their significance with respect to milk and dairy products. Int. J. Food Microbiol. 197, 77–87.

Edalatian, M.R., Habibi Najafi, M.B., Mortazavi, S.A., Alegría, Á., Delgado, S., Bassami, M.R., Mayo, B., 2012. Production of bacteriocins by *Enterococcus* spp. isolated from traditional, Iranian, raw milk cheeses, and detection of their encoding genes. Eur. Food Res. Technol. 234, 789–796.

Fotou, K., Tzora, A., Voidarou, C., Alexopoulos, A., Plessas, S., Avgeris, I., Bezirtzoglou, E., Akrida-Demertzi, K., Demertzis, P.G., 2011. Isolation of microbial pathogens of subclinical mastitis from raw sheep's milk of Epirus (Greece) and their role in its hygiene. Anaerobe 17, 315–319.

Frantzen, C.A., Kot, W., Pedersen, T.B., Ardö, Y.M., Broadbent, J.R., Neve, H., Hansen, L. H., Dal Bello, F., Østlie, H.M., Kleppen, H.P., 2017. Genomic characterization of dairy associated *Leuconostoc* species and diversity of leuconostocs in undefined mixed mesophilic starter cultures. Front. Microbiol. 8, 132.

Fugaban, J.I.I., Holzapfel, W.H., Todorov, S.D., 2021. Probiotic potential and safety assessment of bacteriocinogenic *Enterococcus faecium* strains with antibacterial activity against *Listeria* and vancomycin-resistant enterococci. Curr. Res. Microb. Sci. 2, 100070.

Gaglio, R., Couto, N., Marques, C., Lopes, M., de, F.S., Moschetti, G., Pomba, C., Settanni, L., 2016. Evaluation of antimicrobial resistance and virulence of enterococci from equipment surfaces, raw materials, and traditional cheeses. Int. J. Food Microbiol. 236, 107–114.

Gantzias, C., Lappa, I.K., Aerts, M., Georgalaki, M., Manolopoulou, E., Papadimitriou, K., De Brandt, E., Tsakalidou, E., Vandamme, P., 2020. MALDI-TOF MS profiling of non-starter lactic acid bacteria from artisanal cheeses of the Greek island of Naxos. Int. J. Food Microbiol. 323, 108586.

Garmasheva, I.L., Oleschenko, L.T., 2023. Screening of bacteriocin-producing dairy *Enterococcus* strains using low-cost culture media. Front. Microbiol. 14, 1168835.

Ghrairi, T., Frere, J., Berjeaud, J.M., Manai, M., 2008. Purification and characterisation of bacteriocins produced by *Enterococcus faecium* from Tunisian rigouta cheese. Food Control 19, 162–169.

Giraffa, G., 2003. Functionality of enterococci in dairy products. Int. J. Food Microbiol. 88, 215–222.

Gonzales-Barron, U., Gonçalves-Tenório, A., Rodrigues, V., Cadavez, V., 2017. Foodborne pathogens in raw milk and cheese of sheep and goat origin: a meta-analysis approach. Curr. Opin. Food Sci. 18, 7–13.
Güley, Z., Fallico, V., Cabrera-Rubio, R., Cotter, P.D., Beresford, T., 2022. Identification of *Streptococcus infantarius* subsp. *infantarius* as the species primarily responsible for acid production in Izmir Brined Tulum Cheese from the Aegean Region of Türkiye. Food Res. Int. 160, 111707.
Hammad, A.M., Hassan, H.A., Shimamoto, T., 2015. Prevalence, antibiotic resistance and virulence of *Enterococcus* spp. in Egyptian fresh raw milk cheese. Food Control 50, 815–820.
Hemme, D., Foucaud-Scheunemann, C., 2004. *Leuconostoc*, characteristics, use in dairy technology and prospects in functional foods. Int. Dairy J. 14, 467–494.
Hill, D., O'Connor, P.M., Altermann, E., Day, L., Hill, C., Stanton, C., Ross, R.P., 2020. Extensive bacteriocin gene shuffling in the *Streptococcus bovis/Streptococcus equinus* complex reveals gallocin D with activity against vancomycin resistant enterococci. Sci. Rep. 10, 13431.
Irlinger, F., Yung, S.A.Y.I., Sarthou, A.S., Delbès-Paus, C., Montel, M.C., Coton, E., Coton, M., Helinck, S., 2012. Ecological and aromatic impact of two Gram-negative bacteria (*Psychrobacter celer* and *Hafnia alvei*) inoculated as part of the whole microbial community of an experimental smear soft cheese. Int. J. Food Microbiol. 153, 332–338.
Jeon, H.H., Kim, K.H., Chun, B.H., Ryu, B.H., Han, N.S., Jeon, C.O., 2017. A proposal of *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* subsp. nov. and reclassification of *Leuconostoc mesenteroides* subsp. *suionicum* (Gu et al., 2012) as *Leuconostoc suionicum* sp. nov. based on complete genome sequences. Int. J. Syst. Evol. Microbiol. 67, 2225–2230.
Kondyli, E., Svarnas, C., Samelis, J., Katsiari, M.C., 2012. Chemical composition and microbiological quality of ewe and goat milk of native Greek breeds. Small Rumin. Res. 103, 194–199.
Koort, J., Coenye, T., Vandamme, P., Sukura, A., Björkroth, J., 2004. *Enterococcus hermanniensis* sp. nov., from modified-atmosphere-packaged broiler meat and canine tonsils. Int. J. Syst. Evol. Microbiol. 54, 1823–1827.
Kousta, M., Mataragas, M., Skandamis, P., Drosinos, E.H., 2010. Prevalence and sources of cheese contamination with pathogens at farm and processing levels. Food Control 21, 805–815.
Lauková, A., Tomáška, M., Kmeť, V., Strompfová, V., Pogány Simonová, M., Dvorožňáková, E., 2021. Slovak local ewe's milk lump cheese, a source of beneficial *Enterococcus durans* strain. Foods 10, 3091.
Lianou, A., Samelis, J., 2014. Addition to thermized milk of *Lactococcus lactis* subsp. *cremoris* M104, a wild, novel nisin A–producing strain, replaces the natural antilisterial activity of the autochthonous raw milk microbiota reduced by thermization. J. Food Prot. 77, 1289–1297.
Lianou, D.T., Michael, C.K., Vasileiou, N.G.C., Petinaki, E., Cripps, P.J., Tsilipounidaki, K., Katsafadou, A.I., Politis, A.P., Kordalis, N.G., Ioannidi, K.S., Gougoulis, D.A., Trikalinou, C., Orfanou, D.C., Fragkou, I.A., Liagka, D.V, Tzora, A., Albenzio, M., Mavrogianni, V.S., Caroprese, M.C., Fthenakis, G.C., 2021a. Extensive countrywide field investigation of somatic cell counts and total bacterial counts in bulk tank raw milk in goat herds in Greece. J. Dairy Res. 88, 307–313.
Lianou, D.T., Petinaki, E., Cripps, P.J., Gougoulis, D.A., Michael, C.K., Tsilipounidaki, K., Skoulakis, A., Katsafadou, A.I., Vasileiou, N.G.C., Giannoulis, T., Voidarou, C., Mavrogianni, V.S., Caroprese, M., Fthenakis, G.C., 2021b. Antibiotic resistance of staphylococci from bulk-tank milk of sheep flocks: prevalence, patterns, association with biofilm formation, effects on milk quality, and risk factors. Biology (Basel) 10, 1016.
Lianou, D.T., Skoulakis, A., Michael, C.K., Katsarou, E.I., Chatzopoulos, D.C., Solomakos, N., Tsilipounidaki, K., Florou, Z., Cripps, P.J., Katsafadou, A.I., Vasileiou, N.G.C., Dimoveli, K.S., Bourganou, M.V, Liagka, D.V, Papatsiros, V.G., Kontou, P.I., Mavrogianni, V.S., Caroprese, M., Petinaki, E., Fthenakis, G.C., 2022. Isolation of *Listeria ivanovii* from bulk-tank milk of sheep and goat farms - from clinical work to bioinformatics studies: prevalence, association with milk quality, antibiotic susceptibility, predictors, whole genome sequence and phylogenetic relatioships. Biology (Basel) 11, 871.
Litopoulou-Tzanetaki, E., Tzanetakis, N., 2014. The microfloras of traditional Greek cheeses. Microbiol. Spectrum 2 (1). https://doi.org/10.1128/microbiospec.CM-0009-2012. CM-0009-2012.
Litopoulou-Tzanetaki, E., 1990. Changes in numbers and kinds of lactic acid bacteria during ripening of Kefalotyri cheese. J. Food Sci. 55, 111–113.
Malissiova, E., Papadopoulos, T., Kyriazi, A., Mparda, M., Sakorafa, C., Katsioulis, A., Katsiaflaka, A., Kyritsi, M., Zdragas, A., Hadjichristodoulou, C., 2017. Differences in sheep and goats milk microbiological profile between conventional and organic farming systems in Greece. J. Dairy Res. 84, 206–213.
Margalho, L.P., van Schalkwijk, S., Bachmann, H., Sant'Ana, A.S., 2020. Enterococcus spp. in Brazilian artisanal cheeses: Occurrence and assessment of phenotypic and safety properties of a large set of strains through the use of high throughput tools combined with multivariate statistics. Food Control 118, 107425.
Martin, N.H., Evanowski, R.L., Wiedmann, M., 2023. Invited review: redefining raw milk quality—evaluation of raw milk microbiological parameters to ensure high-quality processed dairy products. J. Dairy Sci. 106, 1502–1517.
McDonald, W.L., Fry, B.N., Deighton, M.A., 2005. Identification of *Streptococcus* spp. causing bovine mastitis by PCR–RFLP of 16S–23S ribosomal DNA. Vet. Microbiol. 111, 241–246.
Millet, L., Saubusse, M., Didienne, R., Tessier, L., Montel, M.C., 2006. Control of *Listeria monocytogenes* in raw-milk cheeses. Int. J. Food Microbiol. 108, 105–114.
Montel, M.C., Buchin, S., Mallet, A., Delbes-Paus, C., Vuitton, D.A., Desmasures, N., Berthier, F., 2014. Traditional cheeses: rich and diverse microbiota with associated benefits. Int. J. Food Microbiol. 177, 136–154.
Morandi, S., Silvetti, T., Miranda Lopez, J.M., Brasca, M., 2015. Antimicrobial activity, antibiotic resistance and the safety of lactic acid bacteria in raw milk Valtellina Casera cheese. J. Food Saf. 35, 193–205.
Moreno, M.R.F., Sarantinopoulos, P., Tsakalidou, E., De Vuyst, L., 2006. The role and application of enterococci in food and health. Int. J. Food Microbiol. 106, 1–24.
Morgan, F., Massouras, T., Barbosa, M., Roseiro, L., Ravasco, F., Kandarakis, I., Bonnin, V., Fistakoris, M., Anifantakis, E., Jaubert, G., Raynal-Ljutovac, K., 2003. Characteristics of goat milk collected from small and medium enterprises in Greece, Portugal and France. Small Rumin. Res. 47, 39–49.
Nami, Y., Vaseghi Bakhshayesh, R., Mohammadzadeh Jalaly, H., Lotfi, H., Eslami, S., Hejazi, M.A., 2019. Probiotic properties of *Enterococcus* isolated from artisanal dairy products. Front. Microbiol. 10, 300.
Nelli, A., Venardou, B., Skoufos, I., Voidarou, C., Lagkouvardos, I., Tzora, A., 2023. An insight into goat cheese: the tales of artisanal and industrial Gidotyri microbiota. Microorganisms 11, 123.
Oikonomou, G., Addis, M.F., Chassard, C., Nader-Macias, M.E.F., Grant, I., Delbès, C., Bogni, C.I., Le Loir, Y., Even, S., 2020. Milk microbiota: what are we exactly talking about? Front. Microbiol. 11, 60.
Özkan, E.R., Demirci, T., Akın, N., 2021a. In vitro assessment of probiotic and virulence potential of *Enterococcus faecium* strains derived from artisanal goatskin casing Tulum cheeses produced in central Taurus Mountains of Turkey. LWT 141, 110908.
Özkan, E.R., Öztürk, H.İ., Demirci, T., Akın, N., 2021b. Detection of biofilm formation, virulence factor genes, antibiotic-resistance, adherence properties, and some beneficial properties of cheese origin *S. infantarius, S. gallolyticus*, and *S. lutetiensis* strains belonging to the *S. bovis/S. equinus* complex. LWT 150, 112077.
Papadimitriou, K., Anastasiou, R., Mavrogonatou, E., Blom, J., Papandreou, N.C., Hamodrakas, S.J., Ferreira, S., Renault, P., Supply, P., Pot, B., 2014. Comparative genomics of the dairy isolate *Streptococcus macedonicus* ACA-DC 198 against related members of the *Streptococcus bovis/Streptococcus equinus* complex. BMC Genomics 15, 1–21 [Electronic Resource].
Parapouli, M., Delbès-Paus, C., Kakouri, A., Koukkou, A.I., Montel, M.C., Samelis, J., 2013. Characterization of a wild, novel nisin a-producing *Lactococcus* strain with an *L. lactis* subsp. *cremoris* genotype and an *L. lactis* subsp. *lactis* phenotype, isolated from Greek raw milk. Appl. Environ. Microbiol. 79, 3476–3484.
Parente, E., Ricciardi, A., Zotta, T., 2020. The microbiota of dairy milk: a review. Int. Dairy J. 107, 104714.
Perin, L.M., Moraes, P.M., Viçosa, G.N., Júnior, A.S., Nero, L.A., 2012. Identification of bacteriocinogenic *Lactococcus* isolates from raw milk and cheese capable of producing nisin A and nisin Z. Int. Dairy J. 25, 46–51.
Pexara, A., Solomakos, N., Sergelidis, D., Angelidis, A.S., Govaris, A., 2016. Occurrence and antibiotic resistance of enterotoxigenic *Staphylococcus aureus* in raw ovine and caprine milk in Greece. Dairy Sci. Technol. 96, 345–357.
Pitkälä, A., Koort, J., Björkroth, J., 2008. Identification and antimicrobial resistance of *Streptococcus uberis* and *Streptococcus parauberis* isolated from bovine milk samples. J. Dairy Sci. 91, 4075–4081.
Poyart, C., Quesne, G., Trieu-Cuot, P., 2002. Taxonomic dissection of the *Streptococcus bovis* group by analysis of manganese-dependent superoxide dismutase gene (*sod*A) sequences: reclassification of '*Streptococcus infantarius* subsp. *coli*'as *Streptococcus lutetiensis* sp. nov. and of *Streptococcus bovis* biotype II.2 as *Streptococcus pasteurianus* sp. nov. Int. J. Syst. Evol. Microbiol. 52, 1247–1255.
Psomas, E., Sakaridis, I., Boukouvala, E., Karatzia, M.A., Ekateriniadou, L.V, Samouris, G., 2023. Indigenous lactic acid bacteria isolated from raw Graviera cheese and evaluation of their most important technological properties. Foods 12, 370.
Quigley, L., O'Sullivan, O., Stanton, C., Beresford, T.P., Ross, R.P., Fitzgerald, G.F., Cotter, P.D., 2013. The complex microbiota of raw milk. FEMS Microbiol. Rev. 37, 664–698.
Rai, R., Tamang, J.P., 2022. In vitro and genetic screening of probiotic properties of lactic acid bacteria isolated from naturally fermented cow-milk and yak-milk products of Sikkim. India. World J. Microbiol. Biotechnol. 38, 25.
Ralovich, B., 1989. Methods to determine virulence of *Listeria* strains. Int. J. Food Microbiol. 8, 269–272.
Rausch, T., Fritz, M.H.Y., Untergasser, A., Benes, V., 2020. Tracy: basecalling, alignment, assembly and deconvolution of sanger chromatogram trace files. BMC Genom. 21, 1–9 [Electronic Resource].
Ricciardi, A., Storti, L.V, Zotta, T., Felis, G.E., Parente, E., 2020. Analysis of *rpo*B polymorphism and PCR-based approaches for the identification of *Leuconostoc mesenteroides* at the species and subspecies level. Int. J. Food Microbiol. 318, 108474.
Rodríguez, E., González, B., Gaya, P., Nuñez, M., Medina, M., 2000. Diversity of bacteriocins produced by lactic acid bacteria isolated from raw milk. Int. Dairy J. 10, 7–15.
Rosa, N.M., Penati, M., Fusar-Poli, S., Addis, M.F., Tola, S., 2022. Species identification by MALDI-TOF MS and gap PCR–RFLP of non-aureus *Staphylococcus, Mammaliicoccus*, and *Streptococcus* spp. associated with sheep and goat mastitis. Vet. Res. 53, 1–10.
Ruiz, P., Pérez-Martín, F., Seseña, S., Palop, M.L., 2016. Seasonal diversity and safety evaluation of enterococci population from goat milk in a farm. Dairy Sci. Technol. 96, 359–375.
Ruppitsch, W., Nisic, A., Hyden, P., Cabal, A., Sucher, J., Stöger, A., Allerberger, F., Martinović, A., 2021. Genetic diversity of *Leuconostoc mesenteroides* isolates from traditional Montenegrin brine cheese. Microorganisms 9, 1612.
Sameli, N., Sioziou, E., Bosnea, L., Kakouri, A., Samelis, J., 2021. Assessment of the spoilage microbiota during refrigerated (4°C) vacuum-packed storage of fresh Greek Anthotyros whey cheese without or with a crude enterocin A-B-P-containing extract. Foods 10, 2946.

Samelis, J., Kakouri, A., 2022. Microbiological characterization of Greek Galotyri cheese PDO products relative to whether they are marketed fresh or ripened. Fermentation 8, 492.
Samelis, J., Lianou, A., Kakouri, A., Delbes, C., Rogelj, I., Bogovič-Matijašić, B., Montel, M.C., 2009. Changes in the microbial composition of raw milk induced by thermization treatments applied prior to traditional Greek hard cheese processing. J. Food Prot. 72, 783–790.
Sánchez-Juanes, F., Teixeira-Martín, V., González-Buitrago, J.M., Velázquez, E., Flores-Félix, J.D., 2020. Identification of species and subspecies of lactic acid bacteria present in spanish cheeses type "Torta" by MALDI-TOF MS and *pheS* gene analyses. Microorganisms 8, 301.
Schlegel, L., Grimont, F., Ageron, E., Grimont, P.A.D., Bouvet, A., 2003. Reappraisal of the taxonomy of the *Streptococcus bovis/Streptococcus equinus* complex and related species: description of *Streptococcus gallolyticus* subsp. *gallolyticus* subsp. nov., *S. gallolyticus* subsp. *macedonicus* subsp. nov. and *S. gallolyticus* subsp. *pasteurianus* subsp. nov. Int. J. Syst. Evol. Microbiol. 53, 631–645.
Skoufos, I., Giannenas, I., Karamoutsios, A., Tsinas, A., Papadopoulos, G.K., Tzora, A., 2017. Milk quality characteristics of indigenous sheep breeds Boutsko, Frisarta and Karagouniko. J. Hell. Vet. Med. Soc. 68, 59–66.
Solomakos, N., Govaris, A., Angelidis, A.S., Pournaras, S., Burriel, A.R., Kritas, S.K., Papageorgiou, D.K., 2009. Occurrence, virulence genes and antibiotic resistance of *Escherichia coli* O157 isolated from raw bovine, caprine and ovine milk in Greece. Food Microbiol. 26, 865–871.
Terzić-Vidojević, A., Veljović, K., Begović, J., Filipić, B., Popović, D., Tolinački, M., Miljković, M., Kojić, M., Golić, N., 2015. Diversity and antibiotic susceptibility of autochthonous dairy enterococci isolates: are they safe candidates for autochthonous starter cultures? Front. Microbiol. 6, 954.
Tilocca, B., Costanzo, N., Morittu, V.M., Spina, A.A., Soggiu, A., Britti, D., Roncada, P., Piras, C., 2020. Milk microbiota: characterization methods and role in cheese production. J. Proteom. 210, 103534.
Tsafrakidou, P., Sameli, N., Bosnea, L., Chorianopoulos, N., Samelis, J., 2021. Assessment of the spoilage microbiota in minced free-range chicken meat during storage at 4°C in retail modified atmosphere packages. Food Microbiol. 99, 103822.
Tsanasidou, C., Asimakoula, S., Sameli, N., Fanitsios, C., Vandera, E., Bosnea, L., Koukkou, A.I., Samelis, J., 2021. Safety evaluation, biogenic amine formation, and enzymatic activity profiles of autochthonous enterocin-producing Greek cheese isolates of the *Enterococcus faecium/durans* group. Microorganisms 9, 777.
Tsigkrimani, M., Bakogianni, M., Paramithiotis, S., Bosnea, L., Pappa, E., Drosinos, E.H., Skandamis, P.N., Mataragas, M., 2022a. Microbial ecology of artisanal Feta and Kefalograviera cheeses, part I: bacterial community and its functional characteristics with focus on lactic acid bacteria as determined by culture-dependent methods and phenotype microarrays. Microorganisms 10, 161.
Tsigkrimani, M., Panagiotarea, K., Paramithiotis, S., Bosnea, L., Pappa, E., Drosinos, E., Skandamis, P., Mataragas, M., 2022b. Microbial ecology of sheep milk, artisanal Feta, and Kefalograviera cheeses. Part II: technological, safety, and probiotic attributes of lactic acid bacteria isolates. Foods 11, 459.
Tsuda, H., Kodama, K., 2021. Evaluating the technological properties of lactic acid bacteria in Wagyu cattle milk. J. Dairy Res. 88, 210–216.
Vandera, E., Kakouri, A., Koukkou, A.I., Samelis, J., 2019. Major ecological shifts within the dominant nonstarter lactic acid bacteria in mature Greek Graviera cheese as affected by the starter culture type. Int. J. Food Microbiol. 290, 15–26.
Vandera, E., Parapouli, M., Kakouri, A., Koukkou, A.I., Hatziloukas, E., Samelis, J., 2020. Structural enterocin gene profiles and mode of antilisterial activity in synthetic liquid media and skim milk of autochthonous *Enterococcus* spp. isolates from artisan Greek Graviera and Galotyri cheeses. Food Microbiol. 86, 103335.
Vandera, E., Tsirka, G., Kakouri, A., Koukkou, A.I., Samelis, J., 2018. Approaches for enhancing in situ detection of enterocin genes in thermized milk, and selective isolation of enterocin-producing *Enterococcus faecium* from Baird-Parker agar. Int. J. Food Microbiol. 281, 23–31.
Voidarou, C., Tzora, A., Malamou, O., Akrida-Demertzi, K., Demertzis, P.G., Vassos, D., Rozos, G., Alexopoulos, A., Plessas, S., Stavropoulou, E., 2011. Chemical and microbiological characterization of artisan inoculants used for the fermentation of traditional dairy products in Epirus area (Greece). Anaerobe 17, 354–357.
Williams, E.N., Van Doren, J.M., Leonard, C.L., Datta, A.R., 2023. Prevalence of *Listeria monocytogenes, Salmonella* spp., Shiga toxin-producing *Escherichia coli*, and *Campylobacter* spp. in raw milk in the United States between 2000 and 2019: a systematic review and meta-analysis. J. Food Prot. 86, 100014.
Wouters, J.T.M., Ayad, E.H.E., Hugenholtz, J., Smit, G., 2002. Microbes from raw milk for fermented dairy products. Int. Dairy J. 12, 91–109.
Yoon, Y., Lee, S., Choi, K.H., 2016. Microbial benefits and risks of raw milk cheese. Food Control 63, 201–215.
Yuan, L., Sadiq, F.A., Burmølle, M., Wang, N.I., He, G., 2019. Insights into psychrotrophic bacteria in raw milk: a review. J. Food Prot. 82, 1148–1159.
Zhang, H., Feng, Y., LI, X., LUO, J., Ling, W., Zhou, Y., Yong, Y.A.N., Wang, X., LI, H., 2020. Detection of antimicrobial resistance and virulence-related genes in *Streptococcus uberis* and *Streptococcus parauberis* isolated from clinical bovine mastitis cases in northwestern China. J. Integr. Agric. 19, 2784–2791.
Zheng, J., Wittouck, S., Salvetti, E., Franz, C.M.A.P., Harris, H.M.B., Mattarelli, P., O'toole, P.W., Pot, B., Vandamme, P., Walter, J., 2020. A taxonomic note on the genus *Lactobacillus*: description of 23 novel genera, emended description of the genus *Lactobacillus* Beijerinck 1901, and union of *Lactobacillaceae* and *Leuconostocaceae*. Int. J. Syst. Evol. Microbiol. 70, 2782–2858.
Zoumpopoulou, G., Papadimitriou, K., Alexandraki, V., Mavrogonatou, E., Alexopoulou, K., Anastasiou, R., Georgalaki, M., Kletsas, D., Tsakalidou, E., Giaouris, E., 2020. The microbiota of Kalathaki and Melichloro Greek artisanal cheeses comprises functional lactic acid bacteria. LWT 130, 109570.
Zdragas, A., Papadopoulos, T., Mitsopoulos, I., Samouris, G., Vafeas, G., Boukouvala, E., Ekateriniadou, L., Mazaraki, K., Alexopoulos, A., Lagka, V., 2015. Prevalence, genetic diversity, and antimicrobial susceptibility profiles of *Staphylococcus aureus* isolated from bulk tank milk from Greek traditional ovine farms. Small Rumin. Res. 125, 120–126.