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Research article

Biotype-based validation of wild *Leuconostoc mesenteroides* strain genotypes from raw/thermized milks of native Epirus sheep breeds selected and preliminarily applied as cheese adjuncts

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Abstract: Thermization widely used to improve the microbiological quality of raw milk intended for traditional cheese making also inactivates a major part of the beneficial mesophilic LAB biota, particularly wild *Leuconostoc* spp. In this study, thirteen *Leuconostoc mesenteroides* isolates from counterpart raw/thermized (65 ℃; 30 sec) milks of native Epirus sheep breeds, previously identified by 16S rRNA gene sequencing and at the subspecies according to their multiplex-PCR (*rpoB*, *araA*, *dsr*, *sorA*) profiles, were biochemically characterized. The isolates formed seven biotypes, with only one assigned to the subspecies *mesenteroides* and none to *cremoris*. Five biotypes shared an atypical *dextranicum* (*dsr/sorA*) genotype and one biotype seemed novel as it was genomically assignable to the subspecies *jonggajibkimchii* (*araA/dsr*). Six biotypes comprised strains with a strong-positive or positive leucine arylamidase activity, but their valine and cystine arylamidase activities were weak or absent. All except two isolates were β-galactosidase-positive whereas only two typical *mesenteroides* isolates from thermized milk were α-galactosidase-positive. All isolates fermented lactose, but were weak acidifiers in skimmed milk; 46% were dextran-negative. None was β-hemolytic, or possessed β-glucoronidase, trypsin or α-chymotrypsin activity, or formed histamine or tyramine. Excluding their inherent resistance to vancomycin, all biotypes comprised antibiotic susceptible strains. Only one strain was resistant to ampicillin, and five strains were partially susceptible to ciprofloxacin. Two raw sheep milk strains, KFM7 (atypical *dextranicum*; relatively more aciduric and proteolytic *in vitro*) and KFM9 (*jonggajibkimchii*; bacteriocinogenic in milk) previously selected and applied as adjuncts to produce a novel semi-industrial Kefalotyri cheese type from thermized Epirus sheep milk were confirmed to be the most promising biotypes for onsite commercial use in cheese production.

Keywords: *Leuconostoc mesenteroides*; raw sheep milk; thermization; antibiotic resistance; adjuncts

1. Introduction

Mild thermization treatments (57 to 68 ℃ for 10 to 30 sec) applied to improve the microbial quality and safety of raw milk prior to traditional cheese processing [1,2] simultaneously select for thermoduric enterococci and streptococci while inactivate a major part of the multi-functional mesophilic LAB biota comprising mainly wild *Lactococcus* and *Leuconostoc* spp. [3,4]. Particularly, *Leuconostoc (Ln.) mesenteroides*, *Ln. lactis* and *Ln. citreum* are frequent contaminants of raw milks [4], subdominant members of the *Lactococcus (Lc.) lactis* variants in undefined mesophilic cheese starter cultures [5,6], as well as, amongst the most frequent LAB species in artisanal raw milk cheeses [7–9]. *Ln. mesenteroides* can even predominate in fresh or ripened artisan cheeses [10–12].

The use of *Leuconostoc* in dairy technology has a positive impact on product quality and safety [13]. Industrial strains of *Ln. mesenteroides* subsp. *cremoris* are included in commercial cheese starter cultures [14]. Although wild *Leuconostoc* strains usually represent a subdominant fraction of the total LAB biota in raw milk cheeses [9] including starter-free Greek cheeses [15,16], they exert multi-functional beneficial activities during fermentation and ripening. Among others, they produce lactate, acetate and CO_2 contributing to natural cheese curd acidification and 'eye-formation', catabolize citrate, produce acetaldehyde, acetoin, diacetyl and other aromatic compounds, polysaccharides, and vitamins [10,13,17], while many strains produce bacteriocins [18,19] and thus act as biopreservatives and/or potential probiotics [20]. Generally, because food safety and human health concerns associated with dairy leuconostocs are scarce [13,21], the deliberate addition of *Leuconostoc* as costarter or adjunct cultures in traditional cheeses from pasteurized or thermized milks is of great biotechnological interest, with most selected strains originating from raw milk or naturally acidified milk products [22–24], or from artisanal starter-free cheeses being the most enriched natural pools [25,26].

In traditional Greek dairies, mild thermization treatments of raw sheep and goat milks are preferred to pasteurization in order to assure cheese safety with minimum collateral heat damage to milk components and reduced inactivation of the desirable LAB diversity in the milk post-thermally [3]. In this context, *Leuconostoc* spp. were prevalent (48.4% of the isolates) amongst the total LAB biota of chilled raw sheep milks from native Epirus breeds sampled in a local cheese plant. However, their prevalence declined by 42.7% after thermization (65 ℃; 30 sec), displaying the lowest (1.3%) survival amongst all LAB groups isolated by Samelis et al. [27]. Particularly in two raw milk batches displaying an inherent antilisterial activity, *Ln. mesenteroides* strains prevailed [28], but *Leuconostoc* isolates were sporadic in the counterpart thermized milks intended for traditional Kefalotyri cheese production because of the high selective survival of enterococci on all LAB isolation agar media [27]. Similarly, no *Leuconostoc* spp. were isolated from mildly thermized (60 or 67 ℃ for 30 sec) sheep/goat (90:10) milk mixtures dominated by thermoduric enterococci (72%), despite *Ln. mesenteroides* subsp. *mesenteroides* and *Ln. lactis* were frequent (18.4%) in those raw mixed milks before thermization [3].

Ideally, the entire non-starter LAB diversity of raw Epirus sheep milk should be restored after thermization, particularly the beneficial *Leuconostoc* fraction, in order for the desirable sensorial properties of Greek raw milk cheeses [15] to be well-preserved compared to industrially pasteurized milk cheeses [3]. Strategies to develop complex and safe natural starter cultures (NSCs) from raw

sheep milk, without applying heating, were recently described and validated for application in artisan cheese making [29,30]. Particularly regarding traditional Greek hard cheese production, Bosnea et al. [31] recently developed a novel semi-industrial Kefalotyri-type cheese using thermized milk from native Epirus sheep breeds inoculated with a basic NSC comprising one *Streptococcus thermophilus* dairy starter strain and two wild *Lc. lactis* strains, and a native mesophilic adjunct culture comprising one *Lactiplantibacillus plantarum* aromatic strain and, for first time in Greece, two *Ln. mesenteroides* strains representing wild genotypes, one atypical of the subspecies *dextranicum* and one novel of the subspecies *jonggajibkimchii* that prevailed in Epirus raw sheep milk [28]. Considering that the use of dairy *Leuconostoc* as costarter or adjunct cultures in cheese is unlikely to be unsafe [6,13,26], our preliminary selection of raw sheep milk *Ln. mesenteroides* strains for application as Kefalotyri cheese adjuncts [31] was fairly random, although based on their atypical/novel genotyping and the fact that none was β-hemolytic or possessed *vanA/vanB* or transmissible enterococcal virulence genes [28].

Because the novel Kefalotyri-type experimental cheese fermented and ripened with the aid of selected *Ln. mesenteroides* adjunct strains was safe and graded of 'excellent quality', like the control NSC cheese while differing in its volatilome (VOC) profile [31], this study aimed to biochemically characterize and evaluate basic technological and safety aspects of our recent *Ln. mesenteroides* strain collection, including the two adjunct strains already applied. For this purpose, the sugar fermentation patterns, milk acidifying and enzymatic activities, potential bacteriocin production and antilisterial activity in milk, and antibiotic resistance (AR) profiles at the subspecies and the strain biotype level of the wild *Ln. mesenteroides* genotypes originating from raw/thermized milks of native Epirus sheep breeds were determined. The primary objective of this study was to validate the most promising strains for inclusion in complex NSCs for onsite commercial use in traditional Greek cheese technologies.

2. Materials and methods

2.1. Sheep milk isolates, reference (control) strains and culture conditions

Ten wild *Ln. mesenteroides* strains previously isolated from two batches of raw sheep milk (RM1, RM2) of native Epirus breeds and identified at the species level by 16S rRNA gene sequencing [28] were selected for further study (Table 1), along with three sporadic *Leuconostoc* isolates (coded KTM14, KTM15 and KTM18) recovered from RM1 after thermization (65 °C; 30 s; batch TM1) [27].

Three reference *Ln. mesenteroides* ACA-DC (0231, 0493, 0750) strains originating from traditional, starter-free Greek cheeses, kindly provided by Prof. E. Tsakalidou, Laboratory of Dairy Research, Agricultural University of Athens, and previously confirmed to represent distinct genotypes at the subspecies level (S2/*dextranicum*; S4/*mesenteroides*; S5/atypical *dextranicum*) by multiplex PCR [28] as detailed in Table 1, were used as controls for strain biotyping. The multiplex-PCR profiles corresponding to the type strains of the *Ln. mesenteroides* subspecies *cremoris* and *jonggajibkimchii*, not available in our laboratory, and the previous genotypic assignment of the *Ln. mesenteroides* sheep milk isolates to the subspecies, according to Ricciardi et al. [32], are detailed in Table 1, too.

Table 1. Raw sheep milk isolates and reference (control) strains of *Leuconostoc mesenteroides* included in this study and their 16S rRNA gene-based species identification and multiplex PCR-based subspecies discrimination[1].

Strain code/origin	Origin/Isolation Source	Closest relative strain in BLAST	16S rRNA gene seq. similarity (%)	Multiplex-PCR profiles	Multiplex PCR-based strain genotype	Multiplex PCR-based subspecies identification
Raw sheep milk isolates	Raw milk batch					
KFM2, KFM7	RM1	MN229548	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
KFM3	RM1	NR040818	100	*rpoB/araA/dsr*	S3	*jonggajibkimchii*
KFM9	RM1	MN173332	100	*rpoB/araA/dsr*	S3	*jonggajibkimchii*
KFM10	RM1	JN853602	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
KFM31, KFM38	RM2	CP020731	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
KFM36	RM2	MN994413	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
KFM39	RM2	CP065995	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
KFM40	RM2	KT124572	100	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*
Reference (control) strains						
Ln. mesenteroides subsp. *cremoris* ATCC 19254^T	Dairy	NA	NA	*rpoB*	S1	*cremoris*
Ln. mesenteroides subsp. *dextranicum* ACA-DC 0493	Arseniko cheese	Not reported	Not reported	*rpoB/dsr*	S2	*dextranicum*
Ln. mesenteroides subsp. *jonggajibkimchii* DRC 1506^T	Kimchi	NA	NA	*rpoB/araA/dsr*	S3	*jonggajibkimchii*
Ln. mesenteroides subsp. *mesenteroides* ACA-DC 0750	Anthotyro cheese	Not reported	Not reported	*rpoB/araA/dsr/sorA*	S4	*mesenteroides*
Ln. mesenteroides subsp. *dextranicum* ACA-DC 0231	Feta cheese	Not reported	Not reported	*rpoB/dsr/sorA*	S5 (atypical)	Atypical *dextranicum*

[1] All data for the ten raw sheep milk isolates and the multiplex PCR data for the reference ACA-DC strains are adapted from Sioziou et al. [28]. The multiplex PCR-based subspecies discrimination of the species *Ln. mesenteroides* and particularly the profiles of the type *cremoris* and *jonggajibkimchii* strains ATCC 19254^T and DRC 1506^T, respectively, are according to Ricciardi et al. [32].

Three non-*Leuconostoc* strains, the primary natural starter *Streptococcus thermophilus* ST1, the wild, nisin A-producing (NisA+) costarter *Lc. lactis* subsp. *cremoris* M78, and the ripening adjunct strain *Lactiplantibacilus plantarum* H25 [33] previously applied in pilot-scale production of Galotyri PDO cheeses [34] as well as constituents of the mixed NSC applied in the semi-industrial Kefalotyri cheeses microbiologically and physicochemically analyzed by Bosnea et al. [31], were technologically compared with the sheep milk isolates, as specified in the Results. In particular, *Lp. plantarum* H25 was the primary aromatic strain combined with two of the aforementioned genomically atypical (isolate KFM7 in Table 1) or novel (isolate KFM9 in Table 1) *Ln. mesenteroides* strains from raw Epirus sheep milk [28] as an adjunct culture in the novel Kefalotyri-type cheese trials [31].

All isolates were resuscitated by transferring 0.1 mL of frozen (–30 ℃) stock cultures with 20% (w/v) glycerol, in 10 mL MRS broth (Neogen Culture Media, Heywood, UK), incubated at 30 ℃ for 24 h, and then subcultured twice, as above, except for *S. thermophilus* ST1 always subcultured in M17 broth (Merck, Darmstadt, Germany) at 37 ℃. The second subculture was streaked for growth on MRS agar (Neogen) or M17 agar (Merck) at 30 ℃ for 48–72 h, to check and ensure purity by transferring one single colony to new tubes containing 10 mL of MRS or M17 broth incubated at 30 ℃ or 37 ℃, for use in the experiments.

2.2. Biochemical characterization of the Leuconostoc mesenteroides sheep milk isolates

All sheep milk isolates and the ACA-DC strains were rechecked for their gram-positive and catalase-negative reactions and for basic phenotypic traits (i.e., microscopic appearance, CO_2 production from glucose; ammonia from arginine; growth at 4 ℃, 10 ℃, 37 ℃ and 45 ℃; growth in 4% and 6.5% salt; slime formation from sucrose; growth on kanamycin aesculin azide [KAA] agar) to confirm biochemically their 16S rRNA-based identification as members of the genus *Leuconostoc.* Next, all isolates were tested for the fermentation of 13 basic (key) sugars, L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose, and xylose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany), in 96-well mini-plates to identify them biochemically at the species, and obtain potential strain biotypes within each *Ln. mesenteroides* subspecies (Table 1), according to the identification criteria in the Bergey's Manual of Systematic Bacteriology [35] and later taxonomic studies [36]. Strain biotyping was based on the basic (key) sugar fermentation reactions and the ability of the isolates to produce slime (dextran) from sucrose; the biotype-code groups defined by Sameli et al. [37] were followed.

2.3. Primary technological and safety properties of the Leuconostoc mesenteroides sheep milk isolates

2.3.1. Milk acidification capacity

All sheep milk isolates and the control strains were tested for their ability to ferment milk in pure culture *in vitro*. The acidifying activity rate was evaluated by measuring the pH declines (ΔpH) of inoculated (1% v/v) 10% reconstituted skimmed milk (RSM; Neogen) cultures. For this purpose, one set of triplicate 10-mL RSM tubes for each tested strain were simultaneously inoculated as above from the same overnight MRS broth culture and then transferred in an incubator at 37 ℃. All RSM cultures were grown at 37 ℃ for 6 h, shifted to 30 ℃ for up to 24 h, and finally to 22 ℃ for an additional 24 h, in simulation of the gradual temperature reductions of the fresh curds during

traditional cheese processing. On each of the above incubation time intervals, one of the triplicate tubes per strain was removed (i.e., the first one after 6 h at 37 ℃) for sampling, and the remaining two tubes were shifted to the next incubation temperature for the subsequent two samplings at 24 h and 48 h, respectively. In this manner, the use of single 10 mL RSM sample tubes all across the incubation period that would cause breaking of the milk curd, if any, and insertion of abundant external air in each statically growing RSM culture due to vortexing on each sampling interval was avoided, and thus growth continuation of the *Ln. mesenteroides* strains in RSM was not interrupted. The pH of all RSM cultures was measured by direct immersion of the glass electrode of a 3510 digital pH meter (Jenway, Essex, UK) in the milk transferred to a clean 25-mL universal tube. During incubation, the clotting strength and the uniformity of the curd, if any, were recorded macroscopically before vortexing each tube for the pH measurement. This acidification test was done in duplicate for each *Ln. mesenteroides* strain in two independent occasions; the two pH measurements were averaged and compared with the respective pH values of the NSC strains, ST1, M78, and H25, grown under the same conditions in RSM. Additionally, both *Ln. mesenteroides* strains KFM7 and KFM9 previously applied as adjuncts in the experimental Kefalotyri cheeses [31] were comparatively tested with the NSC strains, as above, after their constant growth in RSM at 37 ℃, 30 ℃, or 22 ℃ for 48 h, and were sampled at 24 h and 48 h, respectively.

2.3.2. Antagonistic activity—Challenge tests against *Listeria monocytogenes* in milk

Fresh (30 ℃; 24 h) MRS broth cultures were streaked, or serially diluted, on M17, MRS and Milk Plate Count (MPCA) (Neogen) agar plates. Following growth at 30 ℃ for 48 h, all plates were screened for direct antagonistic activity against the target strain *Listeria monocytogenes* no.10 by the simple agar overlay technique [28]. For the isolates causing inhibition halos, bacteriocin-like activity was tested by the spot-on-lawn and well diffusion assays: filter-sterilized MRS culture (30 ℃; 24 h) cell-free supernatants (CFS) were assayed directly or after adjustment to pH 6.2, heating at 80 ℃ for 15 min, and treatment with 1 mg/mL of proteinase K and trypsin (Sigma Aldrich Chemie GmbH, Steinheim, Germany) at 37 ℃ for 3 to 24 h, as described by Sioziou et al. [28].

Next, the antimicrobial activity of the *Ln. mesenteroides* sheep milk isolates found to produce bacteriocin-like substances (BLIS) in MRS broth was confirmed against 10 reference strains of the species *Listeria monocytogenes* (Scott A, no.10, ISS G79, ISS G185, WSLC 1294, WSLC 1482), *Listeria innocua* (LMG 11387), and *Staphylococcus aureus* (ISS 465, ISS 464, ISS 23) previously used as indicators of the anti-pathogenic activity of the NisA+ *Lc. lactis* subsp. *cremoris* M78/M104 strain genotype [3]. Details on their origin, isolation source, serotype or enterotoxin type [3] are tabulated in the Results. Additionally, one natural *L. monocytogenes* strain survivor from an acid-curd artisan Galotyri PDO cheese and three gram-negative reference strains, *Escherichia coli* ATCC 25922 and ATCC 43888 and *Salmonella* Typhimurium ATCC 700408, served as indicators. All pathogenic strains were cultured in Tryptic Soy broth with 0.6% yeast extract (TSBYE; Neogen) at 30 ℃ (for *Listeria*) and 37 ℃ (for the other species) overnight and then inoculated in the MPCA well assay plates, which were filled with 60 μL CFS (acidic vs. pH-adjusted) of the producer strain in each well, incubated at 30 ℃, and checked for inhibitory zone (in mm) formation, as before [28]. The antimicrobial activity of the *Ln. mesenteroides* BLIS producers was compared with that of the NisA+ activity of strain M78 used as a positive control [3].

Additional RSM challenge experiments were conducted to confirm the ability of antagonistic *Ln.*

mesenteroides strains to produce BLIS with *in situ* antilisterial activity in milk. For this purpose, one set of triplicate 10 mL RSM tubes were co-inoculated with the producer strain and the target strain *L. monocytogenes* no.10 at ca. 6 log CFU/mL and 3.0 log CFU/mL, respectively. The RSM cocultures were divided in three racks, incubated statically at 37 ℃, 30 ℃, or 22 ℃ for 48 h, respectively, and were analyzed microbiologically and for pH at 0 h (after inoculation) and 48 h. Serial decimal dilutions were made in 9-mL quarter-strength Ringer (Neogen) solution and the survival/growth of *L. monocytogenes* no.10 was evaluated by spread-plating on Palcam agar (Neogen) plates, incubated at 30 ℃ for 48 h. Occasionally, the predominant growth of the BLIS producers was confirmed on MRS agar (Neogen) plates incubated at 30 ℃ for 48–72 h. Colony counts were converted to log CFU/mL and the D-log values (log CFU/mL at 48 h—log CFU/mL at 0 h) were determined. After measuring their final (48-h) pH as above, all RSM cocultures were evaluated for manifestation of direct *in situ* antilisterial activity by a modified well diffusion assay (acidic vs. pH-adjusted RSM samples, both heated at 80 ℃ for 15 min before filling the wells) against *L. monocytogenes* no.10, as previously described by Sioziou et al. [28]. The BLIS-negative *Ln. mesenteroides* of the same biotypes/genotypes and the NSC strains (ST1, M78, and H25) were challenged, as above, too, for comparison of their antilisterial activity in milk.

2.3.3. Enzymatic activity

All *Ln. mesenteroides* sheep milk isolates were assayed for 19 constitutive enzymes in their fresh cell biomass using the API ZYM semi-quantitative micro-method, according to the manufacturer instructions (BioMerieux, Marcy l' Etoile, France).

2.3.4. Biogenic amine formation

All sheep milk isolates were also evaluated for their *in vitro* ability to produce histamine and tyramine by using the improved biogenic amine (BA) screening method of Bover-Cid and Holzapfel [38]. BA production was recorded by the color change of the improved BA broth from yellowish to blue or purple, as previously described by Tsanasidou et al. [33]. Additional BAs were not evaluated during this screening study because it is unlikely a food-grade LAB (gram-positive) strain to be unsafe due to BA formation *in situ* in cheese, without being able to form histamine and/or tyramine *in vitro*, the two most important BAs in (dairy) foods [38].

2.3.5. Hemolytic activity

All sheep milk isolates and the ACA-DC strains were retested for hemolytic activity on ready-to-use 5% sheep blood agar plates (Bioprepare, Greece), incubated aerobically at 37 ℃ for 48–72 h. The β-hemolytic reference strains *E. faecalis* ATCC 29212 and *E. faecium* 315VR served as positive controls, as before [33].

2.3.6. Antibiotic susceptibility

All *Ln. mesenteroides* isolates were screened by the Kirby-Bauer disk diffusion method for resistance to eight antibiotics (μg per disk): Ampicillin (AMP; 10), Chloramphenicol (CHL; 30), Ciprofloxacin (CIP; 5), Erythromycin (ERY; 15), Gentamicin (GEN; 10), Penicillin G (PEN; 10 units

per disk), Tetracycline (TET; 30), and Vancomycin (VAN; 30), as described by Tsanasidou et al. [33]. Briefly, two MRS agar plates were spread-inoculated with 100 μL of fresh (24-h) MRS broth cultures of each tested isolate, the inocula were left to absorb for 5–10 min, and then four ready-to-use antibiotic disks (BioMaxima SA, Lublin, Poland) were placed over the cell lawn on each plate. All plates were incubated at 37 ℃ overnight. On the next morning, the diameter of the inhibition zones formed around each disk was measured with a micrometer (model D15, Mitutoyo, Kanagawa, Japan). The above process was repeated once on a different day with new fresh *Ln. mesenteroides* cultures, the two measurements were averaged, and the zone results were interpreted as sensitive (S), intermediate (I), or resistant (R), according to the breakpoints recommended by CLSI [39]. Because the CLSI M100 does not publish *Leuconostoc*-specific interpretive breakpoints, those specified for *Enterococcus* sp. [39] and used in our laboratory since the 2018 BIOTRUST project [33] were borrowed as the most related genus coexisting with *Ln. mesenteroides* in raw sheep milk [3,28] and traditional Greek cheeses [15]. On this basis, *E. faecalis* ATCC 29212 and *E. faecium* 315VR served as positive controls in the present antibiotic susceptibility tests, too, as before [33]. Additional clarifications regarding the use of MRS agar instead of Mueller-Hinton agar for the disk diffusion assays and the reproducibility of the AR data in accordance with the relevant literature are provided in the Results.

3. Results and discussions

3.1. Biotyping of the Leuconostoc mesenteroides sheep milk isolates at and within the subspecies level

All sheep milk isolates produced gas (CO_2) from glucose but not ammonia from arginine, and grew abundantly in MRS broth with 4% and 6.5% salt, also at 10 ℃ and 37 ℃, but not at 4 ℃ or 45 ℃, or on KAA agar. Moreover, 53.9% of them (7/13 isolates) formed abundant slime (dextran) when cultured on modified MRS agar with 5% sucrose, as only the ACA-DC 0231 from the *Ln. mesenteroides* control strains did (Figure 1). Slime (dextran) formation was a critical technological trait for discriminating the *Ln. mesenteroides* sheep milk isolates at the subspecies [35,36] and biotype level, in combination with their basic sugar fermentation profiles also presented in Figure 1.

In a previous polyphasic taxonomic study, Sameli et al. [37] distinguished the diverse psychrotrophic *Ln. mesenteroides* spoilage biota of refrigerated, vacuum-packed Anthotyros whey cheese in 12 strain biotypes (L1A to L1F, L2A to L2C, L3, L4A, and L4B) at and within the subspecies level, as detailed in Figure 1. Using the same taxonomic approach, Sioziou et al. [28] found that 80% (8/10) of the present *Ln. mesenteroides* KFM isolates shared the atypical S5 profile (*rpoB/dsr/sorA*) of the *Ln. mesenteroides* subsp. *dextranicum* ACA-DC 0231 strain (Table 1). The remaining two isolates (KFM3, KFM9) shared the S3 profile (*rpoB/araA/dsr*) of the type strain DRC 1506^T of *Ln. mesenteroides* subsp. *jonggajibkimchii* [32] originally found in kimchi [36] and recently in fermented dairy products [23,40,41]. None of the wild *Ln. mesenteroides* raw sheep milk strain genotypes shared any of the respective profiles of the type strains of the subspecies *cremoris* (ATCC 19254^T; *rpoB*), *dextranicum* (DSM 20484^T; *rpoB/dsr*), or *mesenteroides* (ATCC 8293^T; *rpoB/araA/dsr/sorA*) [32]; hence, none matched the reference strains ACA-DC 0493 and ACA-DC 0750 which possessed the latter two profiles, respectively (Table 1) [28].

In this study, the biotyping procedure of Sameli et al. [37] was followed (Figure 1) and the results were highly consistent with regard to: (i) the two unique *jonggajibkimchii* raw milk isolates KFM3 and KFM9 shared the oligofermenting L4A biotype profile, which comprises slime-producing

strains unable to ferment D-xylose, like the single WM108 isolate from Anthotyros cheese [37]; (ii) none of the remaining eight KFM isolates matched phenotypically the subsp. *dextranicum* ACA-DC 0493 (S2 genotype) or the subsp. *mesenteroides* ACA-DC 0750 (S4 genotype) (Table 1; Figure 1), confirming their genotypic multiplex PCR distinction [28]; (iii) instead, the typical/S2 *dextranicum* ACA-DC 0493 strain clustered alone in the new biotype L1X, whereas the typical/S4 *mesenteroides* ACA-DC 0750 strain clustered in biotype L1C, intermixed with an atypical *jonggajibkimchii* strain WM137 [37]; of note, the ACA-DC 0750 strain originated from an Anthotyros cheese, too (Table 1).

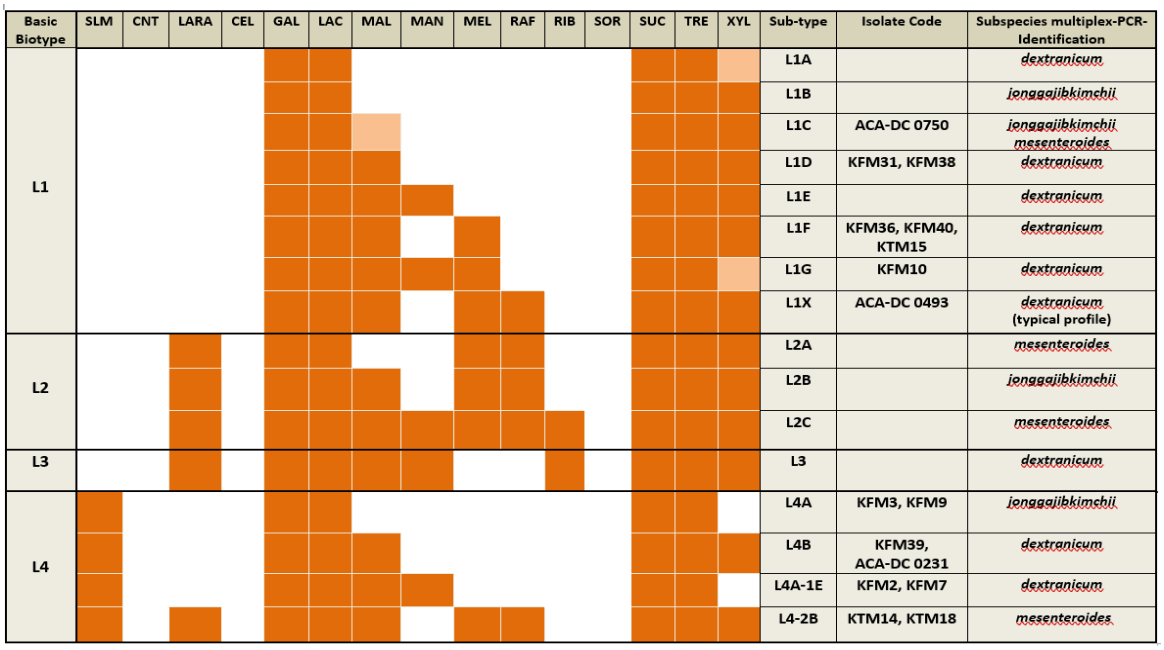


Figure 1. Biotyping based on key sugar fermentation patterns and dextran-producing ability (slime; SLM) of *Leuconostoc mesenteroides* strains isolated from counterpart raw (KFM isolates) or thermized (KTM isolates) sheep milk of native Epirus breeds. Red-brown boxes indicate acid production from L-arabinose (LARA), cellobiose (CEL), galactose (GAL), lactose (LAC), maltose (MAL), mannitol (MAN), melibiose (MEL), raffinose (RAF), ribose (RIB), sorbitol (SOR), sucrose (SUC), trehalose (TRE), and xylose (XYL). Light-pink boxes indicate a weak positive fermentation reaction. CNT, No acid production in MRS broth base without sugar. Uncoloured boxes, negative fermentation reaction.

The above findings, apart from confirming that the ACA-DC 0493 and 0750 strains are biochemically and molecularly distinct, highlight that several dairy strains of the subspecies *mesenteroides* are phenotypically and genetically intermixed with the DRC 1506^T type strain of the subspecies *jonggajibkimchii*, whose description is solely based on this single strain from kimchi [36], as criticized by Ricciardi et al. [32]. However, neither the ACA-DC 0493 nor the ACA-DC 0750 formed slime (dextran) (Figure 1) despite all typical *dextranicum*, *mesenteroides* and *jonggajibkimchii* strains are dextran-producers, unlike the subspecies *cremoris* which cannot produce dextran [35,36]. Nevertheless, atypical dextran-negative *Ln. mesenteroides* strains belonging to the former two subspecies are frequent isolates from dairy, meat and plant foods [7,32,42], while the *Ln. mesenteroides* slime-forming ability may be lost after strain sub-culturing for many sequent times [35].

Notably, from the eight KFM isolates sharing the atypical S5 *dextranicum* genotypic profile, only KFM39 clustered with the slime-producing ACA-DC 0231 in biotype L4B; the remaining seven KFM isolates were distributed in an additional four distinct biotypes, two previously detected in Anthotyros cheese (L1D and L1F) [37] and two newly defined ones (L1G and L4A-1E) (Figure 1). Particularly the new intermediate biotype L4A-1E comprised the duplicate RM1 isolates KFM2 and KFM7 of one reference strain in BLAST (Table 1); both produced slime and were unable to ferment D-xylose, like biotype L4A, but fermented maltose and mannitol, like biotype L1E (Figure 1) from Anthotyros cheese [37]. The fourth new biotype L4-2B comprised sporadic *Ln. mesenteroides* survivors from TM1 (KTM14 and KTM18), which were typical subsp. *mesenteroides* strains [35,42]. Both produced slime and were the only sheep milk isolates that fermented L-arabinose (Figure 1) and shared the S4 (*rpoB/araA/dsr/sorA*) *mesenteroides* profile. Conversely, the third KTM15 isolate shared the atypical *dextranicum* S5 genotype and the respective L1F biotype with two RM2 isolates, KFM36 and KFM40 (Figure 1).

In summary, the high biotype variability in Figure 1 confirmed the high phenotypic and genotypic intra-species heterogeneity of *Ln. mesenteroides* [35], further extended to high intra-subspecies heterogeneity. Therefore, the subspecies discrimination of *Ln. mesenteroides* dairy isolates (with the exception of the subspecies *cremoris*) is unfeasible by several methods, singly or in combination, including 16S rRNA sequencing [10,43], whole-cell protein patterns or ribotyping [42], MALTI-TOF analysis [16,40,44,45], RAPD fingerprinting [7,46], or multiplex PCR profiling [32]. Consequently, nowadays, whole genome sequencing (WGS) and advanced transcriptomic analyses are required to assure the exact subspecies designation of our wild *Ln. mesenteroides* strains and provide insights into their genomic and metabolic features and roles in milk fermentations [43].

3.2. Primary safety properties of the diverse Leuconostoc mesenteroides strain biotypes

None of the sheep milk isolates was β-hemolytic, in agreement with our previous testing [28], the genus description [35] and recent studies focusing on *Ln. mesenteroides* strains of raw milk or cheese origin [20,24,47,48]. Also, none produced tyramine or histamine in improved BA broth with amino acid precursors, similarly to all tested *Ln. mesenteroides* isolates from raw camel milk [48] and artisanal Spanish [25], Italian [8], and Brazilian [26] cheeses. Overall, *Leuconostoc* spp./*Ln. mesenteroides* strains are not BA producers [13], but some strains of the subspecies *mesenteroides* were reported to produce tyramine and tryptamine [35]. Thus, although it is unlikely our present strains to produce BAs other than histamine and tyramine, additional phenotypic BA evaluations coupled with screening for relevant amino acid decarboxylase genes and/or HPLC analyses of model cheese milk samples fermented with *Ln. mesenteroides*, with or without supplementation with precursor amino acids, may be needed to fully assure safety of strains commercially applied as Greek cheese adjuncts.

3.3. Antibiotic susceptibility

The phenotypic AR profiles of the *Ln. mesenteroides* sheep milk isolates are presented in Table 2. Although the Mueller-Hinton agar is recommended by CLSI [39] for cultivating bacteria for the Kirby-Bauer disk diffusion test and particularly strains of the genera *Enterococcus*, *Streptococcus*, and *Lactococcus* [39,49], previous relevant studies always use MRS agar for cultivation of *Leuconostoc* and *Lactobacillus* [8,19,20,22,49,50]. Regarding the inhibitory zone diameters (mm) as

far as the *Leuconostoc*-specific interpretive breakpoints are concerned, Morandi and co-workers followed the 2007 CLSI-M100-S17 [8,49] that is earlier from the CLSI-M100S26 used in this study [39], while others used a common breakpoint range for all antibiotics tested: R (resistant) ≤ 15 mm; I (intermediate susceptible) 16–20 mm, and S (susceptible) ≥ 21 mm [19,20,24,48]. Conversely, a very recent study by Yilmaz et al. [50] using MRS agar for the disk diffusion testing of LAB from dairy and seafood sources, too, reports on specific breakpoints for each antibiotic, according to Humphries et al. [51]; as an example, for gentamicin (10 μg/disk) tested herein, but not reported for *Enterococcus* in the CLSI [39], the specific breakpoints are R ≤ 12 mm; I 13–14 mm; S ≥ 15 mm [50], critically differing from the aforementioned common breakpoint range. Therefore, all previous studies above were considered for testing the antibiotic susceptibility of our *Enterococcus*, *Streptococcus* and *Ln. mesenteroides* isolates from raw or thermized sheep milk [27,28], analyzed in parallel; M17 agar vs. Mueller Hinton agar was used for cultivating the *Enterococcus* [52] and *Streptococcus* [53] strains, and solely MRS agar for the present *Ln. mesenteroides* strains (Table 2) because they were unable to promote dense growth on Mueller-Hinton agar in order to display well-defined inhibitory zones of antibiotic susceptibility.

Table 2. Antibiotic susceptibility of the wild *Leuconostoc mesenteroides* isolates from Epirus sheep milk assessed by the disk diffusion method.

Strain biotype[2]	Isolate code	Antibiotic tested (μg/disc)[1]							
		AMP 10	CHL 30	CIP 5	ERY 15	GEN 10	PEN 10U	TET 30	VAN 30
L1C	ACA-DC 0750	S (20.1)	S (23.2)	S (20.9)	S (30.1)	S (18.8)	R (14.2)	S (27.7)	R (6.0)
L1D	KFM31	S (19.7)	S (28.3)	I (17.0)	S (29.9)	S (19.7)	S (15.9)	S (30.1)	R (6.0)
	KFM38	S (25.8)	S (29.1)	I (20.1)	S (31.5)	S (21.8)	S (20.8)	S (31.1)	R (6.0)
L1F	KFM36	S (18.2)	S (26.7)	I (17.5)	S (28.7)	S (17.7)	S (16.5)	S (27.0)	R (6.0)
	KFM40	S (23.6)	S (29.4)	I (19.9)	S (31.7)	S (21.4)	S (16.0)	S (32.0)	R (6.0)
	KTM15	S (26.3)	S (32.8)	S (23.8)	S (34.1)	S (25.7)	S (20.1)	S (35.8)	R (6.0)
L1G	KFM10	R (12.6)	S (30.9)	I (19.2)	S (34.0)	S (21.7)	S (17.9)	S (32.2)	R (6.0)
L1X	ACA-DC 0493	S (18.4)	S (23.3)	S (21.4)	S (26.5)	S (18.3)	S (19.9)	S (24.1)	R (6.0)
L4A	KFM3	S (22.7)	S (30.2)	S (22.3)	S (33.6)	S (23.0)	S (21.1)	S (29.5)	R (6.0)
	KFM9	S (24.5)	S (25.0)	S (24.0)	S (35.0)	S (26.2)	S (25.8)	S (34.5)	R (6.0)
L4B	KFM39	S (25.0)	S (23.8)	S (20.9)	S (32.6)	S (21.6)	S (18.3)	S (32.3)	R (6.0)
	ACA-DC 0231	S (21.9)	S (23.3)	I (19.6)	S (28.6)	S (17.3)	S (18.6)	S (29.5)	R (6.0)
L4A-1E	KFM2	S (19.8)	S (30.0)	S (23.8)	S (30.0)	S (23.0)	S (20.5)	S (29.8)	R (7.0)
	KFM7	S (21.5)	S (26.1)	S (24.0)	S (32.8)	S (22.5)	S (23.3)	S (31.9)	R (7.0)
L4-2B	KTM14	S (26.3)	S (27.8)	S (24.9)	S (28.4)	S (20.9)	S (19.6)	S (30.5)	R (8.1)
	KTM18	S (23.4)	S (34.7)	S (22.5)	S (34.6)	S (23.9)	S (17.7)	S (32.7)	R (6.0)

[1] AMP, ampicillin; CHL, chloramphenicol; CIP, ciprofloxacin; ERY, erythromycin; GEN, gentamicin; PEN, penicillin; TET, tetracycline; VAN, vancomycin. The numbers nearby the antibiotics indicate μg or units (U) per disk. S (susceptible), I (intermediate), R (resistant); the numbers in bracket indicate the zone size (mm). [2] The sheep milk isolates and the reference ACA-DC strains of *Ln. mesenteroides* are sorted in accordance with their assignment to the nine strain biotypes presented in Figure 1.

Based on the breakpoints for enterococci [39] and those reported for gentamicin (10 μg/disk) [50], all *Ln. mesenteroides* isolates, including the three ACA-DC strains, were resistant to vancomycin, irrespective of strain biotype in Figure 1. It is well established that *Leuconostoc* spp./*Ln. mesenteroides* possess phenotypic vancomycin-resistance, a genus-specific intrinsic trait, which is not transmittable to other coexisting bacteria in the (food) niche [13]. However, none was found to possess *vanA* or *vanB* when tested comparatively to numerous co-isolates of enterococci and streptococci from raw sheep milk [28]. Otherwise, except for the KFM10 strain found resistant to ampicillin, all *Ln. mesenteroides* were susceptible to six of the remaining seven antibiotics tested, including gentamicin. Ciprofloxacin was the only antibiotic to which all raw sheep milk strains in biotypes L1D, L1F and L1G displayed intermediate susceptibility, including the ampicillin-resistant KFM10; the rest eight strains and mainly the KFM7 and KFM9 adjunct strains [31] were susceptible (Table 2). The reference *Ln. mesenteroides* strains were also susceptible to all seven antibiotics, except of the ACA-DC 0750 that was resistant to penicillin and the ACA-DC 0231 that was partially susceptible to ciprofloxacin (Table 2).

The antibiotic susceptibility results in Table 2 are in general agreement with previous dairy studies: Excluding their natural intrinsic phenotypic resistance to vancomycin and kanamycin, wild *Ln. mesenteroides* strains were susceptible to all antibiotics tested, including ampicillin, chloramphenicol, erythromycin, gentamicin, penicillin, and tetracycline [8,19,20,22,24,25,48,49] tested herein, too, with some exceptions. For instance, Campedelli et al. [17] sequenced three dairy *Ln. mesenteroides* strains atypically resistant to erythromycin or tetracycline and/or streptomycin.

Also, two bioactive *Ln. mesenteroides* ZB7 and Z8 strains from Algerian camel milk were resistant to streptomycin and tetracycline [19], while a single *Ln. mesenteroides* strain, ZT6, from raw Turkish milk was resistant to eleven antibiotics, including erythromycin and tetracycline [22]. By a MIC-based testing as recommended by EFSA, Kamarinou et al. [47] detected three resistant strains, one to chloramphenicol, one to gentamicin, and one to erythromycin, gentamicin and streptomycin, amongst 25 *Ln. mesenteroides* Greek cheese strains, but none resistant to ampicillin [47], unlike the single strain KFM10 (biotype L1G) from raw sheep milk (Table 2). Overall, ampicillin resistance neither was phenotypically found in any of the above previous studies nor genomically detected in 16 *Ln. mesenteroides* strains from traditional Montenegrin brine cheeses [41]. Most recently, ten ampicillin-resistant *Ln. mesenteroides* subsp. *mesenteroides* strains were reported by Yilmaz et al. [50], but 90% of them were of fish origin and only one strain originated from raw goat milk.

Regarding ciprofloxacin (Table 2), a similar trend of intermediate sensitivity was noted for seven *Ln. mesenteroides* strains from raw cow milk in Morocco [24]. Most importantly, 83% of the wild-type *Leuconostoc* strains from north Italian traditional cheeses, including 14 of total 19 (73.7%) *Ln. mesenteroides* strains, were resistant to ciprofloxacin [8], as also they were two *Ln. mesenteroides* strains, F17 and F18, from raw camel milk with high biotechnological potential [48] and one *Ln. citreum* strain from a Spanish starter-free cheese [25]. Altogether the above findings corroborate that ciprofloxacin, a quinolone-inhibitor of bacterial nucleic acid synthesis, must be regarded amongst the antibiotics to which *Leuconostoc* spp. commonly possess or may develop high resistance. Lastly, it is worth noting that all *Ln. mesenteroides* isolates from Moroccan raw cow milk and artisanal Portuguese Pico cheese were resistant to cephalosporins [24,54].

3.4. Milk acidifying activity

All *Ln. mesenteroides* sheep milk isolates and the ACA-DC strains fermented RSM weakly

during the first 6 h at 37 ℃ and onwards, failing to curdle the milk even after 42 h at their optimal growth temperature (22–30 ℃) range; an exception was the ACA-DC 0750 subspecies *mesenteroides* strain (biotype L1C) which formed a soft 'watery' curd (Table 3). Specifically, the final (48-h) ΔpH values were ≤ 1.0 pH unit, except of the biotype L4A-1E strains (KFM2, KFM7; ΔpH 1.32–1.40), KFM9 (ΔpH 1.19) in biotype L4A, and the ACA-DC cheese strains (ΔpH 1.19–1.27); strain KFM10 (biotype L1G) was the weakest RSM acidifier (ΔpH 0.36) (Table 3). Similarly, only two of 12 selected *Leuconostoc* isolates from an artisanal Brazilian cheese, one assigned to *Ln. mesenteroides* and the other to *Ln. pseudomesenteroides*, stood out in the acidifying activity in 10% RSM, with respective ΔpH values of 1.12 and 1.04 after 24 h at 30 ℃; all the rest showed < 1.0 pH unit reduction [26]. However, it is worth noting that when the two selected cheese adjunct strains KFM7 and KFM9 were grown in 10% RSM constantly at 30 ℃ for 24 h (i.e., without a preceding 6-h growth at 37 ℃ as it was set in the shifting incubation temperature acidification test; Table 3), the ΔpH value of strain KFM7 increased to 1.45 (mean pH 5.22) from 1.27 (mean pH 5.40; Table 3). Conversely, the respective ΔpH value of strain KFM9 was unaffected by the 6-h incubation at 37 ℃ as it was 1.00 (mean pH 5.67) vs. 1.03 (mean pH 5.64/24 h) in Table 3. Nevertheless, apart from confirming the higher acidification capacity of KFM7 than KFM9 at 30 ℃, the above observation was indicative of difficulty by both strains to acidify the RSM at 37 ℃, despite both grew well in MRS broth at 37 ℃ during biotyping (viz. section 3.1). Indeed, when incubated constantly at 37 ℃ for 24 to 48 h, both strains failed to reduce the RSM pH < 6.0, displaying ΔpH (48-h) values as low as 0.39 (KFM 9; mean pH 6.28) and 0.56 (KFM7; mean pH 6.11) compared to the relatively high ΔpH (48-h) values of 1.23 (mean pH 5.44) and 1.53 (mean pH 5.14), and 1.39 (mean pH 5.28) and 1.65 (mean pH 5.02) when the KFM9 and KFM7 growth occurred at 30 ℃ and 22 ℃, respectively (data not tabulated). These findings clearly indicate a strong growth temperature-dependent milk acidifying activity that was almost negligible at 37 ℃ but remarkably enhanced for both strains at 30 ℃ and 22 ℃ due to their mesophilic or even psychrotrophic nature. Indeed, both were amongst the prevalent atypical and/or novel *Ln. mesenteroides* biota isolated from chilled raw sheep milk before traditional Kefalotyri processing in a local plant [28]. Also, based on the results, their optimal growth temperature likely is within 20–30 ℃, a biotechnological trait that should be reconsidered when KFM7, KFM9 or additional *Ln. mesenteroides* strains of this collection will be reapplied as hard cheese adjuncts [31]. Eventually, the adjunct strain KFM7 was confirmed as the best milk acidifier at 30 ℃ and 22 ℃ amongst all present *Ln. mesenteroides* strains, causing moderate clotting with entrapped CO_2 release inside the RSM curd at 30 ℃, but not at 22 ℃, after 48 h, whereas no curd or visible gas was formed by KFM9 in RSM under the above conditions.

Overall, the above findings confirm that (skimmed) milk is seldom acidified and curdled by *Leuconostoc* and *Ln. mesenteroides* in particular [8,35,54]. However, two *Ln. mesenteroides* strains, F17 and F18, from camel milk were recently described to possess a rapid and strong milk acidification capability when tested in 10% RSM enriched with 0.3% (v/v) yeast extract and 2% glucose [48]. We validated this by similarly enriching the fermenting RSM cultures at 30 ℃ of the L4A (KFM3, KFM9) and L4A-1E (KFM2, KFM7) strain biotypes. All enriched RSM cultures curdled firmly and their pH fell to 4.40–4.56 after an additional 24-h growth at 30 ℃ (data not tabulated); these pH values were an additional 0.63 to 1.04 pH units lower than those measured after constant growth at 30 ℃ for 48 h. Evidently, the *in vitro* acidification capacity of both strain biotypes in enriched RSM increased at comparable levels with their fresh cultures (pH 4.49–4.61) grown at 30 ℃ for 24 h in normal MRS broth containing 2% glucose. Therefore, the fastidious growth requirements

of *Leuconostoc*/*Ln. mesenteroides* [35] are not adequately satisfied, unless the (skimmed) milk is enriched with glucose and essential nutrients present in the rich MRS formula. Wild *Ln. mesenteroides* strains with a strong milk acidifying capacity are rarely isolated as, for instance, a novel *Ln. mesenteroides* subsp. *jonggajibkimchii* YS7-10 strain from an Indian hard-variety yak-milk *chhurpi*, which acidified 10% RSM to pH 4.5 without glucose or other enrichment [23].

Table 3. Acidifying activity of the wild *Leuconostoc mesenteroides* isolates from Epirus sheep milk, in comparison with the reference ACA-DC strains, in reconstituted skim milk (RSM) at gradually decreasing incubation temperatures[1].

Strain biotype[2]	Isolate code	Shifting incubation (temperature/time) conditions					
		37 ℃ for an initial 6 h		30 ℃ for an additional 18 h		22 ℃ for an additional 24 h	
		RSM pH	Milk curd[3]	RSM pH	Milk curd	RSM pH	Milk curd
L1C	ACA-DC 0750	6.40	−	5.44	(+)	5.40	(+)
L1D	KFM31	6.17	−	5.85	−	5.77	−
	KFM38	6.39	−	5.99	−	5.77	−
L1F	KFM36	6.37	−	6.10	−	5.93	−
	KFM40	6.21	−	5.76	−	5.67	−
	KTM15	6.19	−	5.82	−	5.82	−
L1G	KFM10	6.40	−	6.32	−	6.31	−
L1X	ACA-DC 0493	6.45	−	5.47	−	5.40	−
L4A	KFM3	6.40	−	5.67	−	5.67	−
	KFM9	6.48	−	5.64	−	5.48	−
L4B	KFM39	6.40	−	5.73	−	5.67	−
	ACA-DC 0231	6.05	−	5.48	−	5.48	−
L4A-1E	KFM2	6.41	−	5.75	−	5.35	−
	KFM7	6.38	−	5.40	−	5.27	−
L4-2B	KTM14	6.46	−	6.05	−	6.01	−
	KTM18	5.99	−	5.83	−	5.78	−
Basic NSC strains							
S. thermophilus ST1		4.99	++	4.08	++	4.00	++
Lc. lactis/cremoris M78		5.48	−/(+)	4.66	++	4.46	++
Lp. plantarum H25		6.19	−	5.47	−	5.23	−

[1] Each pH value is the mean of two RSM replicates; the SD values (narrowly ranged from 0.01 to 0.07) are not shown for table simplification); the initial pH of RSM was 6.67 ± 0.11. [2] The sheep milk isolates and the reference ACA-DC strains of *Ln. mesenteroides* are sorted in accordance with their assignment to the nine strain biotypes presented in Figure 1. [3] Type of milk curd: ++ hard, strong milk clotting; + soft, moderate milk clotting; (+) partially and weakly clotted milk; − no evidence of milk clotting/curd formation.

Conversely, *S. thermophilus* ST1 grew abundantly in RSM, managing a ΔpH value as high as 1.68 after 6 h at 37 ℃, which increased further to 2.59 pH units after 24 h at 30 ℃ and resulted in strong curd formation (Table 3). The NisA+ costarter strain *Lc. lactis* M78 was more aciduric than all *Ln. mesenteroides* biotypes, too. In contrast to the ST1 and M78 basic starter strains, *Lp. plantarum* H25 failed to acidify (<pH 5.2) and curdle the RSM (Table 3) despite the pH of its MRS culture (30 ℃, 48 h)

was 3.85. This condition was improved when incubation at 37 ℃ and 30 ℃ was extended from 6 and 18 h (Table 3) to 24 and 48 h, respectively, but still the final pH of the pure H25 cultures in RSM remained around 5.0 and the milk clotted softly (data not shown). However, to our surprise, *Lp. plantarum* H25 totally failed to acidify (mean pH 5.52) and curdle the RSM under constant growth at 22 ℃ for 48 h, despite its mesophilic nature, and unlike *Ln. mesenteroides* KFM7 which, as mentioned, curdled the RSM by reducing its pH to 5.0 after 48 h at 22 ℃. These findings have important practical implications because they corroborate with the high prevalence (ca. 8.5 log CFU/g) of the adjunct strain H25 in the novel ripening Kefalotyri-type cheese post-fermentation vs. a more delayed subdominant growth (ca. 7.0–7.5 log CFU/g) of the KFM7 + KFM9 adjunct strains accompanied by 'eye opening' by the middle (day 18) to the end (day 30) of ripening [31], attributed primarily to the KFM7 strain now; based on this study, the adjunct culture constituent H25 + KFM7 + KFM9 strains were compatible and performed well in succession to the basic starter strains in the ripening Kefalotyri-type cheese [31].

3.5. Bacteriocin-mediated antilisterial activity

Consistent with their growth medium-dependent acid formation activity, all *Ln. mesenteroides* sheep milk isolates completely inhibited growth of *L. monocytogenes* no. 10 on the MRS agar (initial pH 5.7 ± 0.1) overlays, but none did so in the respective overlays on M17 agar (initial pH 7.2 ± 0.2) containing 0.5% lactose instead of 2% glucose [28]. However, the novel strain *Ln. mesenteroides* subsp. *jonggagibkimchii* KFM9 (biotype L4A) was the only sheep milk isolate that showed a clear antilisterial activity after streaking (Figure 2A) or decimally diluting it (Figure 2B) on MPCA (initial pH 6.9 ± 0.2; contains 0.1% glucose and 0.1% skimmed milk powder) overlays. The KFM9 inhibitory activity was retained in the acidic MRS CFS tested by the spot-on-lawn (20 μL) method (Figure 2C), as well as in the well assays after CFS neutralization (pH 6.2) and heating (80 ℃; 15 min), but it was lost after treatment with proteolytic enzymes (Figure 2D). Thus, strain KFM9 excreted a heat-resistant antilisterial peptide in MRS broth under optimal growth conditions at 30 ℃. This bacteriocin-like substance (BLIS) most likely represents a known class IIa or IIc bacteriocin, similarly to most Bac+ *Ln. mesenteroides* strains of dairy food origin [18,19,41,55]. To further investigate this, the antimicrobial activity of the CFS (acidic vs. pH-adjusted MRS culture filtrates) of the KFM9 and the control NisA+ M78 strains were comparatively tested under the same well diffusion assay conditions against 14 reference bacterial indicator strains of the pathogenic genera/species *Listeria* spp./*L. monocytogenes*, *Staphylococcus aureus*, *Escherichia coli* and *Salmonella.* The results are summarized in Table 4.

It was confirmed that the neutralized CFS of the KFM9 strain was strongly inhibitory against all seven *L. monocytogenes* indicator strains, including Scott A, and *L. innocua* LMG 11387. Based on the inhibition zone sizes, strain WSLC 1482 from a German soft smear cheese was the most sensitive whereas strain GLLM1 from a mature artisan acid-curd Greek Galotyri PDO cheese was relatively the most resistant to the KFM9 BLIS (Table 4). Conversely, none *S. aureus* indicator strain was inhibited by the neutralized KFM9 CFS, unlike the neutralized nisin-containing CFS of M78 that inhibited all gram-positive indicators. Moreover, as it was anticipated, the antilisterial and anti-staphylococcal activity of M78 was maximized by its acidic CFS due to the well-documented increase of nisin activity at low pH; in most of these cases, a double inhibitory zone was formed around each well, one internal clear zone defining the combined increase in the nisin/acid activity

and one external faint zone due to abundant acid diffusion in the plates. A combined BLIS/acid antilisterial activity was also noted for the KFM9 strain. Moreover, two *S. aureus* indicators were inhibited by the acidic KFM9/CFS while the third enterotoxin C-producing *S. aureus* ISS 23 strain was resistant. Expectedly, neither M78 nor KFM9 inhibited growth of the *E. coli* or *Salmonella* indicators by their neutralized CFS because typical LAB bacteriocins, like nisin, are inactive against Gram-negative bacteria (Table 4).

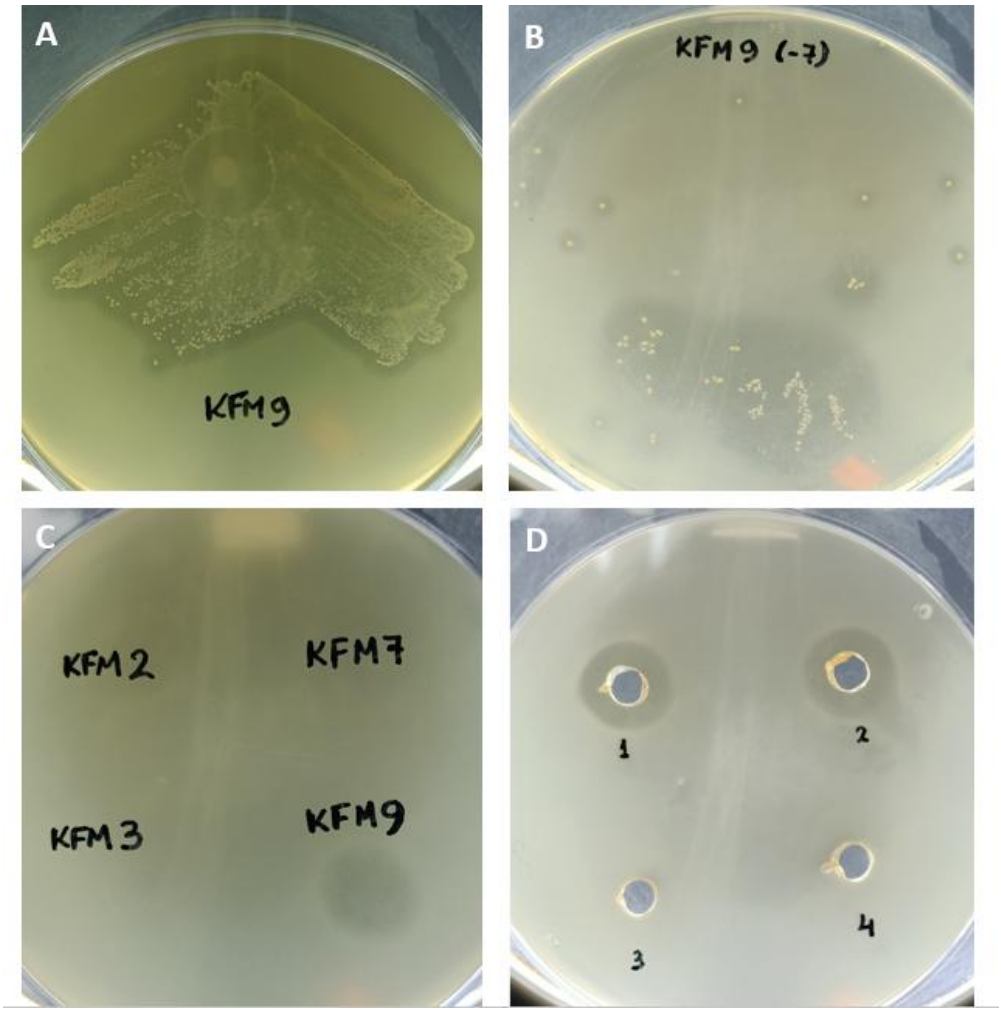


Figure 2. Bacteriocin-mediated activity of the raw sheep milk isolate *Leuconostoc mesenteroides* subsp. *jonggajibkimchii* KFM9 against the target strain *Listeria monocytogenes* no.10: Direct *in vitro* antilisterial activity of the streaked (A) or the serially diluted (B) KFM9 culture grown on milk plate count agar (MPCA), overlayed with a listerial cell lawn and then incubated at 30 ℃ overnight; (C) Spot-on-lawn assay: Growth inhibition of *L. monocytogenes* no.10 on MPCA by the MRS (30 ℃; 24 h) cell-free supernatant (CFS; pH 4.6) of strain KFM9 – the CFS of the KFM2, KFM7 and KFM3 strains caused no inhibition; (D) Well diffusion assay: Wells opened in a solidified MPCA plate pre-seeded with listerial cells were filled with 60 µL of neutralized (pH 6.0–6.2) MRS CFS of strain KFM9 before (well 1) or after (well 2) heating at 80 ℃ for 15, or after treatment with proteinase K (well 3) and trypsin (well 4).

Table 4. Antimicrobial activity of the wild *Leuconostoc mesenteroides* KFM9 isolate from raw Epirus sheep milk (acidic vs. pH-adjusted culture filtrates) evaluated against pathogenic strains by well diffusion assays, in comparison with *Lactococcus lactis* subsp. *cremoris* M78.

Indicators	Source	Serotype	Inhibition by tested strain before and after CFS neutralization			
			M78-CFS Acidic	M78-CFS pH-adjusted	KFM9-CFS Acidic	KFM9-CFS pH-adjusted
Listeria monocytogenes ScottA	Clinical	4b	+/(+) (14.5/28.3)	+ (12.4)	+/(+) (16.3/23.9)	+ (15.8)
L. monocytogenes no.10	Meat	4ab				+ (14.3)
L. monocytogenes ISS G79	Soft cheese	1/2b	+/(+) (15.4/20.8)	+ (12.2)	+ (19.5)	+ (14.8)
L. monocytogenes ISS G185	Blue cheese	1/2a	+/(+) (16.1/22.7)	+ (11.7)	+/(+) (18.2/22.8)	+ (15.9)
L. monocytogenes WSLC 1294	Goat cheese	3b	+/(+) (15.4/19.0)	+ (12.0)	+ (19.3)	+ (15.5)
L. monocytogenes WSLC 1482	Soft cheese	1/2b	+ (25.5)	+ (12.3)	+ (26.5)	+ (17.3)
L. monocytogenes GLLM1	Galotyri PDO	Unknown	+/(+) (9.5/16.0)	+ (9.9)	+ (15.8)	+ (12.9)
Listeria innocua LMG 11387			+ (23.0)	+ (10.8)	+ (21.1)	+ (15.2)
		Enterotoxin type				
Staphylococcus aureus ISS 465	Clinical	A	+/(+) (9.1/18.4)	+ (10.3)	(+) 20.6	−
Staphylococcus aureus ISS 464	Cow cheese	A	+ (11.5)	+ (9.9)	(+) 13.7	−
Staphylococcus aureus ISS 23	Sheep's cheese	C	+ (10.8)	+ (7.8)	−	−
Escherichia coli ATCC 43888	Human feces	Non-Shiga-like toxin producer	+ (17.4)	−	+ (16.8)	−
Escherichia coli ATCC 25922			+ (18.1)	−	+ (17.6)	−
Salmonella Typhimurium ATCC 700408		Phage type DT104	+ (14.1)	−	(+) (22.3)	−

+, clear inhibition zone; (+), faint inhibition zone, −, no inhibition. The zone diameter (mm), inclusive of the 6-mm well, is shown in brackets.

Next, challenge experiments were conducted to estimate whether the KFM9 strain was able to produce BLIS with activity against *L. monocytogenes* no.10 under the same co-culturing conditions in RSM at 37 ℃, 30 ℃, and 22 ℃ (Table 5). The BLIS-negative strains KFM2, KFM7, KFM3, ST1, H25, and the NisA+ strain M78 were tested comparatively as controls. Overall, results showed that all four *Ln. mesenteroides* strains failed to inhibit *L. monocytogenes* growth in all RSM cocultures, with this failure becoming maximal in the 37 ℃/48 h samples that contained listerial populations well above 8 log CFU/mL and thus displayed highly positive D-log values, i.e., around 5-log units (Table 5). All D-log values were ca. 10-fold lower and quite similar at 30 ℃ and 22 ℃ because *L. monocytogenes* growth remained below the 8-log level in most RSM cocultures at both temperatures. Exceptions were the coculture with the KFM7 strain at 22 ℃, which retarded listerial growth below the 7-log level (D-log value 3.44), and the clear exception of the KFM9 coculture at 30 ℃ that displayed a D-log value by 1.04 to 1.77 log units lower than the other seven cocultures, including the counterpart coculture at 22 ℃. Additionally, the *Ln. mesenteroides* KFM9/30 ℃ coculture was the only that displayed a weak but clear

antilisterial activity following neutralization and heating (80 ℃; 15 min) of the 48-h RSM samples (Table 5). Therefore, it was evident that strain KFM9 produced BLIS when grown in milk at 30 ℃, but not at 37 ℃ or 22 ℃. In contrast, the BLIS-negative KFM2, KFM7 and KFM3 strains exerted a weak, acid-mediated *Listeria* inhibition in the well assays at 30 ℃, but not at 37 ℃ or 22 ℃.

Regarding acidification, major decreases in the terminal (48-h) pH of all *Ln. mesenteroides* cocultures with *L. monocytogenes* no.10 (Table 5) compared to their corresponding pure RSM cultures grown under similar incubation conditions (Table 3) were observed. For instance, in the presence (5.85 to 7.20 log units) of *L. monocytogenes* in the KFM9 and KFM7 RSM cocultures at 30 ℃, the terminal (48-h) pH (Table 5) was lower by 0.94 and 1.07, and 0.78 and 0.94 pH units, compared to the pH of their corresponding pure RSM cultures after 24 h at 30 ℃ or after shifting at 22 ℃ for an additional 24 h (Table 3). Much more impressive were the pH reductions in all *Ln. mesenteroides* RSM cocultures at 37 ℃ having terminal (48-h) pH values below 6.0 (Table 5), whereas, as it was highlighted in section 3.4, all had failed to reduce the RSM pH likewise across their growth in pure culture in RSM, either after 6 h (Table 3) or even 48 h. Specifically, while the two adjunct strains KFM9 and KFM7 slightly acidified and thus failed to curdle the RSM after growth in pure culture constantly at 37 ℃ for 48 h (i.e., pH 6.28 and 6.11), both reduced the pH down to 5.20 and 4.77, respectively, in the very high (>8 log units) presence/population density of *L. monocytogenes* cells grown in their RSM cocultures (Table 5) The interpretation of this finding is difficult. Considering the high prevalence (>8 log units) of *Ln. mesenteroides* in all pure cultures or the cocultures (data not shown) and the weak acidifying activity (pH ≥ 6.2 during growth at 37 ℃ to 22 ℃ for 72 h) of pure *L. monocytogenes* no.10 cultures in sterile sheep milk [56], it can be suggested that either the acidifying activity of the growing *Ln. mesenteroides* strains in RSM at 30 ℃ and 37 ℃ was stimulated by the major listerial co-growth and catabolism that resulted in the formation of essential nutrients, or the enhanced acidification of the *Ln. mesenteroides* strains might be due to activation of quorum sensing (cell density-dependent) regulatory mechanisms [57]. Further advanced studies are needed to investigate this, and pan-genomic and transcriptomic analyses to fully characterize the BLIS-forming KFM9 strain, the structural bacteriocin gene/s it possesses and the activity of bacteriocin/s it may produce under real cheese making conditions [43].

Meanwhile, regarding the NSC strains, M78 fully inhibited growth and further caused partial inactivation of *L. monocytogenes* in RSM as revealed by the negative D-log values of the pathogen and the clear antilisterial activity of the neutralized/boiled RSM coculture samples at 30 ℃ and 37 ℃ (Table 5). The primary milk acidifier ST1 fully suppressed *L. monocytogenes* growth at 37 ℃, but this strong acid-mediated inhibitory activity was not retained in neutralized milk, while it was reduced above 100-fold at 30 ℃. *Lp. plantarum* H25 behaved similarly to the BLIS-negative *Ln. mesenteroides* strains in the respective RSM cocultures, failing to inhibit growth of *L. monocytogenes* at 30 ℃ and even more at 37 ℃ (Table 5), while none of the NSC strains was challenged at 22 ℃ in the course of this study.

Table 5. Inhibitory activity of the wild *Leuconostoc mesenteroides* KFM9 strain against *Listeria monocytogenes* no.10 in reconstituted skimmed milk (RSM) in comparison with three BLIS-negative *Ln. mesenteroides* and the basic natural starter culture (NSC) strains at different growth temperatures.

Strain/Parameter tested	*Leuconostoc mesenteroides* strains				NSC strains		
	KFM2	KFM7	KFM3	KFM9	ST1	M78	H25
Challenge at 37 °C/48 h							
Curd formation at 24 h	−	−	−	−	++	−	−
Curd formation at 48 h	−	++	(+)	(+)	++	+	−
Final coculture pH at 48 h	5.89	4.77	5.30	5.20	4.19	5.00	6.00
Survival/growth of Lm10 (log CFU/mL) at 48 h	8.32	8.34	8.44	8.55	2.75	1.28	7.76
D-log values	4.87	4.89	4.99	5.10	−0.70	−2.17	4.31
Antilisterial RSM activity (Acidic/boiled; zone mm)	−	−	−	−	+ 10.5	+ 10.1	−
Antilisterial RSM activity (Neutralized/boiled; mm)	−	−	−	−	−	+ 9.4	−
Challenge at 30 °C/48 h							
Curd formation at 24 h	−	+	(+)	−	+	+	−
Curd formation at 48 h	+	++	++	+	++	++	+
Final coculture pH at 48 h	4.74	4.33	4.41	4.70	4.29	4.42	4.73
Survival/growth of Lm10 (log CFU/mL) at 48 h	7.62	7.20	7.34	5.85	5.93	2.90	6.54
D-log values	4.17	3.75	3.89	2.40	2.48	−0.55	3.09
Antilisterial RSM activity (Acidic/boiled; zone mm)	−/(+) 8.8	(+) 9.9	−/(+) 9.4	+ 10.1	(+) 9.3	+ 10.8	−
Antilisterial RSM activity (Neutralized/boiled; mm)	−	−	−	(+) 9.5	−	+ 9.2	−
Challenge at 22 °C/48 h					Not tested at 22 °C		
Curd formation at 24 h	−	−	−	−			
Curd formation at 48 h	−	−	−	−			
Final coculture pH at 48 h	5.92	5.51	5.60	5.95			
Survival/growth of Lm10 (log CFU/mL) at 48 h	7.39	6.89	7.59	7.38			
D-log values	4.14	3.44	4.14	3.93			
Antilisterial RSM activity (Acidic/boiled)	−	−	−	−			
Antilisterial RSM activity (Neutralized/boiled)	−	−	−	−			

Each challenge test was performed twice and each value is the mean; the SD values (range 0.03–0.13 for the pH and 0.02–0.24 for the *L. monocytogenes* counts) are not tabulated for table simplification; D-log values were calculated as (log CFU/mL at 48 h – log CFU/mL at 0 h); the mean initial Lm10 count was 3.45 ± 0.08 CFU/mL. Symbols for curd formation and antilisterial RSM activity are as previously explained in the footnotes of Tables 3 and 4, respectively.

3.6. Variations in the enzymatic activities of the Leuconostoc mesenteroides sheep milk isolates—Biotype-based adjunct strain selection

Major strain-dependent variations in the API ZYM profiles of the 13 *Ln. mesenteroides* sheep milk isolates occurred in comparison with the NSC strains (Table 6). Domingos-Lopes et al. [54] also reported high strain-dependent variability in the API ZYM profiles of wild *Ln. mesenteroides* isolates from artisanal Pico cheese. Overall, autochthonous starter or adjunct LAB strain candidates should possess strong to moderate acid phosphatase, aminopeptidase, esterase and/or esterase/lipase and primary glycolytic (α-galactosidase and/or β-galactosidase, or α-glucosidase) activities, while they should lack β-glucoronidase and β-glucosidase considered potential mediators of colon carcinogenesis [47,54]. However, at least for the β-glucosidase activity of many LAB, especially *Lp. plantarum*, a beneficial, multi-functional role in food fermentation and human health was reviewed by Paventi et al. [58], supporting our decision to apply *Lp. plantarum* H25, a β-glucosidase-positive strain with a superior enzymatic profile (Table 6), as an adjunct ripening culture in pilot-scale traditional Galotyri PDO [34] and in semi-industrial Kefalotyri cheese trials [31].

Basically, a strong β-galactosidase activity is required for all dairy starter LAB strains, including *S. thermophilus* ST1 (Table 6), as the main enzyme responsible for lactose breakdown to glucose and galactose during cheese milk fermentation [47]. On this basis, except for the KFM38 strain in biotype L1D and the ampicillin-resistant strain KFM10 in biotype L1G, all *Ln. mesenteroides* sheep milk biotypes displayed strong β-galactosidase activity (Table 6). Moreover, nine of them (69%) were positive for α-glucosidase, five (38.5%) were positive or weakly positive for β-glucosidase, whereas their lipolytic and proteolytic enzyme activities generally were absent or weak (Table 6). However, the atypical *dextranicum* biotype L4A-1E (KFM2/KFM7) that was the best milk acidifier also had the best API ZYM profile with higher aminopeptidase activities, particularly a strong positive activity for leucine arylamidase, and weak esterase (C4) activity (Table 6). Conversely, the novel antilisterial subsp. *jonggajibkimchii* KFM9 strain and its close relative strain KFM3 in biotype L4A shared an overall poorer API ZYM profile compared to other biotypes, but both were strongly positive for β-galactosidase and positive for leucine arylamidase, particularly the antilisterial BLIS-forming strain KFM9. Meanwhile, the typical subsp. *mesenteroides* biotype L4-2B from thermized sheep milk showed a similar profile to biotype L4A-1E; both isolates KTM14 and KTM18 lacked esterase activity, but showed promise by being the only *Ln. mesenteroides* strain biotype with α-galactosidase activity, unlike the ST1 and M78 starters (Table 6). Wild *Ln. mesenteroides* isolates from artisanal Greek or Portuguese cheeses having both alpha and beta galactosidase activities were pointed out by Kamarinou et al. [47] and Rampanti et al. [59].

However, strain FMX3 which showed the strongest activity for both galactosidases and was the only BLIS-producer amongst 25 *Ln. mesenteroides* fresh Greek cheese isolates, simultaneously it was the most resistant to erythromycin, gentamicin, and streptomycin, additionally to kanamycin and vancomycin [47], unlike our isolates that were susceptible to erythromycin and gentamicin (Table 2).

Table 6. Enzymatic activity (API ZYM) profiles of *Leuconostoc mesenteroides* isolates from sheep milk of native Epirus breeds before or after thermization[1].

Species	NSC strains [2]			*Leuconostoc mesenteroides* strain biotypes												
				L1D		L1F			L1G	L4A		L4B	L4A-1E		L4-2B	
Strain code	ST1	M78	H25	KFM31	KFM38	KFM36	KFM40	KTM15	KFM10	KFM3	KFM9	KFM39	KFM2	KFM7	KTM14	KTM18
Alkaline phosphatase	0	40	40	20	10	20	10	10	20	10	20	20	30	30	10	20
Esterase (C 4)	20	20	20	0	0	20	0	10	0	0	10	0	20	10	0	0
Esterase Lipase (C 8)	0	30	20	0	0	0	0	0	0	0	0	0	0	0	0	0
Lipase (C 14)	0	0	10	0	0	0	0	0	0	0	0	0	0	0	0	0
Leucine arylamidase	40	40	40	40	20	10	20	40	10	20	30	30	40	40	30	20
Valine arylamidase	10	30	40	0	0	0	0	0	0	0	0	0	10	10	0	0
Cystine arylamidase	10	30	40	0	10	0	10	0	0	0	10	10	20	10	10	10
Trypsin	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-chymotrypsin	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Acid phosphatase	30	40	40	20	30	40	30	30	40	40	30	30	30	30	30	30
Naphthol-AS-BI-Phosphohydrolase	10	40	30	10	20	20	20	30	20	20	20	20	20	20	20	20
α-galactosidase	0	0	40	0	0	0	0	0	0	0	0	0	0	0	30	20
β-galactosidase	40	0	40	40	0	40	40	40	0	40	40	40	40	40	40	40
β-glucuronidase	0	0	20	0	0	0	0	0	0	0	0	0	0	0	0	0
α-glucosidase	0	40	40	0	40	30	40	30	30	0	0	0	30	30	30	30
β-glucosidase	0	0	30	0	30	20	30	0	30	0	0	0	20	0	0	0
N-acetyl-β-glucosaminidase	0	0	40	0	0	0	0	0	0	0	0	0	0	0	0	0
α-mannosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
α-fucosidase	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0

[1] The reactions were graded from 0 (negative) to 5 (strongly positive) based on the change in the color intensity after the addition of the ZYM A & ZYM B reagents in each well. Activity (approximate values) was expressed as arbitrary units of substrate hydrolyzed; 0: Negative (as first-well control); 1: Liberation of 5 nmol; 2: 10 nmol; 3: 20 nmol; 4: 30 nmol; 5: 40 nmol or more. Liberation of 20 to 40 nmol or more was considered a clearly positive enzymatic reaction, and 10 nmol a weak-positive reaction. [2] The natural starter culture (NSC) strains *Streptococcus thermophilus* ST1, *Lactococcus lactis* M78 and *Lactiplantibacillus plantarum* H25 were included for comparison of their enzymatic activities.

Regarding β-glucosidase and the human health concerns associated with this enzyme, it should stressed that all dextran-producing *Ln. mesenteroides* strain biotypes (L4A, L4B, L4A-1E, and L4-2B) comprised β-glucosidase-negative isolates, except for KFM2 that was found weakly positive (Table 6). Particularly the selected KFM7 and the BLIS-producing KFM9 strains preliminarily applied as Kefalotyri cheese adjuncts [31] as well as the KFM39 (L4B) and KTM14/KTM18 (L4-2B) strains can be considered phenotypically safe for further application as novel adjuncts in future pilot-scale cheese studies. However, a complete genomic safety evaluation of all promising strains is required before their application in commercial Greek cheeses. Particularly, the biotype L4-2B (KTM14/KTM18) deserves further study for its α-galactosidase-positive activity, complementing *Lp. plantarum* H25 *in situ* in cheese (Table 6). Whereas the ampicillin-resistant and β-glucosidase-positive strain KFM10, as well as, all β-glucosidase-positive strains in biotypes L1D and L1F should be excluded. Lastly, none of the present *Ln. mesenteroides* sheep milk isolates showed an unwanted β-glucoronidase or trypsin and/or α-chymotrypsin activity (Table 6), a result considered positive for cheese safety.

In summary, the results of this biotyping study validate the preliminary random selection but deliberate use of the two *Ln. mesenteroides* KFM7 and KFM9 strains combined with *Lp. plantarum* H25 as adjuncts for the semi-industrial production of a novel Kefalotyri cheese, which was successful biotechnologically [31]. Indeed, compared to the mature (90-day-old) Kefalotyri cheese made with the basic NSC only, the novel cheese fermented and ripened with the aid of the three adjunct strains contained less residual lactose, more acetate and had an overall less diversified volatilome profile, thanks to the dominance (>8 log CFU/g) of *Lp. plantarum* H25 during ripening. The combined *Ln. mesenteroides* KFM7 and KFM9 (gas-forming, vancomycin-resistant) colony counts increased around 7-log CFU/g in the novel Kefalotyri cheese type; their prevalence was associated with small 'eye' formation and a significant decrease in citrate after the fermentation step and a higher acetate content during ripening. Acetone, butyric acid, methyl butyrate, ethyl ether and ethanol were the most abundant volatile compounds in both cheese types [31]. Overall, the use of selected autochthonous dairy *Leuconostoc* spp./*Ln. mesenteroides* strains able for *in situ* production of optimal gas (CO_2) amounts, VOCs and BLIS in traditional hard cheese technologies is considered to have a positive practical implication, particularly regarding the development of small, evenly distributed 'eyes' in the cheese matrix during fermentation and ripening [8,9,13]. In Greece, the production of Swiss-type cheeses with large shiny eyes formed by the metabolic activity of commercial cultures of propionic acid bacteria (PAB) is very limited, if any, but presence of small holes formed by heterofermentative LAB in Graviera, Kefalograviera, and Kefalotyri cheeses is desired [10,15,31].

4. Conclusions

All β-galactosidase-positive and antibiotic susceptible *Ln. mesenteroides* sheep milk isolates can be considered preliminarily safe for use in artisan-type cheese fermentations and may contribute to milk acidification, eye-formation and complementary enzymatic activities during ripening. Further in-depth research studies combining advanced biochemical and sensory analyses with WGS and transcriptomic analyses of the selected strains are needed to determine the growth pattern and the biotechnological and potential bioprotective and/or probiotic role of the KFM7, KFM9 or additional strain biotypes of the diverse *Ln. mesenteroides* as adjuncts in traditional Greek cheese technologies.

Use of AI tools declaration

The authors declare they have not used Artificial Intelligence (AI) tools in the creation of this article.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Dr John Samelis—Conceptualization, Investigation, Corresponding—Supervision, Compilation, Preparation of first draft, Reviewing, Editing; Athanasia Kakouri—Investigation, Reviewing, Editing.

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