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Biotyping and antibiotic susceptibility of animal streptococci from raw/thermized milks of native Epirus sheep breeds

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Abstract

Aim: Animal streptococci surviving thermization of raw milk prior to traditional Greek cheese processing may include both harmless and potentially harmful strains with multiple antibiotic resistances. Therefore, the aims of this study were to biotype and evaluate the antibiotic resistance and primary technological and safety properties of wild streptococci occurring in Greek sheep milk before and after mild thermization.

Methods: Sixteen *Streptococcus* isolates previously isolated from two raw/thermized (65ºC; 30 s) counterpart milks of native Epirus sheep breeds and identified by 16S ribosomal RNA (rRNA) gene sequencing were biochemically characterized using conventional methods (phenotypic tests, sugar fermentation patterns, API 20 STREP and API ZYM profiles) and assessed for susceptibility to eight antibiotics by the disk diffusion method, and for their milk acidification capacity, bacteriocin activity, enzymatic activity profiles, and biogenic amine (BA) formation in vitro.

Results: Tetracycline-resistant (57% of the total 7 isolates) *Streptococcus parauberis* and ciprofloxacin-resistant *Streptococcus gallolyticus* subsp. *pasteurianus* (1 isolate) and *Streptococcus lutetiensis* (2 isolates) strains occurring in raw milk were not recovered post-thermally. Instead, thermization selected for a tetracycline-resistant (50% of the total 6 isolates) thermophilic group previously genotyped as *Streptococcus equinus*, herein biotyped as *Streptococcus pneumoniae.* None of the *Streptococcus* isolates was resistant to ampicillin, chloramphenicol, erythromycin, gentamycin, penicillin, or vancomycin, or β-hemolytic, or produced histamine and tyramine. The milk acidifying activity rate (final skimmed milk pH 4.79 to 5.47 after growth at 37ºC for 6 h, gradually decreasing to 22ºC for a total of 48 h) of the isolates was species-dependent and increased in the order *S. equinus* > *S. gallolyticus* > *S. lutetiensis* > *S. parauberis*.

Conclusions: Except for the two bacteriocin-producing and α-galactosidase-positive *S. lutetiensis* antilisterial strains (KFM 55 and KFM 60), which show promise, all animal streptococci derived from Epirus sheep milk should be considered *a priori* unsafe for inclusion in complex natural cheese starter cultures because they belong to pathogenic, mastitis-causing species or subspecies. The two promising *S. lutetiensis* strains require further safety evaluations for streptococcal virulence and antibiotic resistance genes, mainly to ciprofloxacin, before their commercial use as natural cheese starters.

Keywords

sheep milk, thermization, antibiotic resistance, animal streptococci, *Streptococcus lutetiensis*, *S. parauberis*, *S. equinus*, *S. gallolyticus*

Introduction

Thermization is the oldest and most commonly used empirical strategy to improve the microbiological quality of raw milk (RM) with minimum collateral heat damage to milk components and restricted undesirable changes in the flavor of milk and milk products compared to pasteurization [1, 2]. Unlike pasteurization (72°C for 15 s or equivalent at 63°C for 30 min), thermization is not precisely defined by law but generally describes a wide range of subpasteurization (< 72°C) treatments of RM (57 to 68°C for 5 s up to 30 min, but usually for 10 to 20 s) which do not cause complete inactivation of alkaline phosphatase [2–4]. However, even mild thermization treatments markedly reduce the counts of spoilage, mainly psychrotrophic gram-negative bacteria (i.e., *Pseudomonas* and *Enterobacteriaceae*), and inactivate low counts or few cells of naturally occurring pathogenic (*Salmonella*, *Escherichia coli*, *Yersinia enterocolitica*, *Staphylococcus aureus*, *Listeria monocytogenes*) bacteria, without fully suppressing the technological RM microbiota primarily consisting of lactic acid bacteria (LAB) [2, 3, 5, 6].

Altogether, the above beneficial effects explain why thermization is empirically preferred to pasteurization in the manufacture of traditional cheeses, either on the basis of national Protected Designation of Origin (PDO) cheese specifications [3] or to extend the keeping quality of milk during chilled storage prior to further processing [4], as it happens in many Greek dairies [5]. The milder the thermization treatment, the lesser the negative impact of heat on milk quality (e.g., denaturation of whey proteins, loss of vitamins, inactivation of lipoprotein lipase and other enzymes involved in milk and cheese flavor development) [2, 3] and the higher the survival of the RM biota in thermized milk (TM), and thus, the greater the natural starter and non-starter LAB diversity in the resultant TM cheeses [4, 5]. However, the technological benefits of thermization are contradicted by certain safety concerns, mainly its ineffectiveness against bacterial spores, and the possibility that some gram-positive, non-sporulating pathogens remain viable [3, 4].

Moreover, unavoidably, thermization selects for thermoduric RM bacterial contaminants, mainly *Enterococcus* and *Streptococcus.* This natural selective survival of enterococci and animal streptococci in TM [5, 6], or their potential prevalence in RM cheeses [7] or in natural starter cultures (NSCs) derived from RM for use in traditional cheese production [8, 9] should be a concern because of the controversial nature and role of these LAB genera in foods [10, 11]. Indeed, numerous strains of *Streptococcus* and *Enterococcus* of animal, fecal, or clinical origin are opportunistic pathogens; they are virulent and antibiotic resistant (AR), can cause β-hemolysis, mastitis in ruminants and many different healthcare-associated infections in humans, and hence, may compromise the safety of RM/TM cheeses and other dairy products [12–14]. In particular, certain species, or several strains within a species, of the thermophilic *Streptococcus equinus/Streptococcus bovis* (SESB) complex and of the mesophilic, pyogenic *S. uberis* group bearing virulence genes and multidrug resistance in response to veterinary treatments on farms [15, 16] can be transferred to RM from teat-infected animals [17] and may survive in TM cheese to cause infection.

On the other hand, certain species or subspecies of the SESB complex, as for instance *Streptococcus gallolyticus* subsp. *macedonicus, S. infantarius*, and *Streptococcus lutetiensis*, comprise multi-functional, safe and promising strains for dairy fermentations [18–21]. However, despite their beneficial role in artisan cheeses, none of the above streptococci is Generally Recognized as Safe (GRAS) or included in the Qualified Presumption of Safety (QPS) list of species, likewise the genus *Enterococcus* entirely [12, 22]; a complete safety evaluation at the strain level is a prerequisite for their potential use in (dairy) foods, according to the current EFSA [22] recommendations. In this context, a novel strategy described by Chessa et al. [8] to develop a freeze-dried NSC by keeping the entire LAB biota of Sardinian sheep RM resulted in a mixture of 33 distinct genotypes belonging to eight non-QPS species, *E. faecium*, *E. durans*, *E. faecalis*, *S. gallolyticus*

subsp. *macedonicus, S. equinus*, *S. lutetiensis*, *S. oralis*, *S. salivarius*, and one QPS species, *Lacticaseibacillus paracasei.* Although all genotypes were considered safe for applying that complex NSC in cheese production [9], different RMs from sheep or other domestic animals may contain virulent or multidrug resistant (MDR) *Enterococcus*, *Streptococcus* or other non-QPS LAB strain contaminants, rendering the resultant NSC concentrates unsafe for use.

Indeed, two RM batches from native Epirus sheep breeds displaying inherent antilisterial activity mainly attributed to safe, enterocin-producing *E. faecium/E. durans* strains also contained *E. faecalis* strains harboring virulence (*gelE*, *ace*) genes [23]. Furthermore, controversial *Streptococcus parauberis* strains [14] were prevalent in one RM batch, suggesting that teat-infected sheep with subclinical mastitis were milked [23]. Because 14 distinct *Enterococcus* spp. strain biotypes, including those with single or multiple enterocin gene activity, became prevalent (83.7%) in the above milks post-thermally [6], evaluation of enterococci was prioritized in order to select the most suitable, antagonistic, antibiotic susceptible and safe strains for inclusion in a complex NSC; the isolation of vancomycin-resistant *E. faecalis* biotype 3B strains from TM was concerning [24]. Meanwhile, the increased survival (8.6%) in TM (65ºC; 30 s) of potentially harmful thermophilic streptococci as opposed to the major declines of lactococci and mesophilic streptococci (22.8%) and leuconostocs (42.7%) [6], required further investigations. However, unlike enterococci and lactococci [5, 24], published data referring to the biochemical, molecular and technological characterization of streptococci found in milk of Greek native sheep breeds are very limited. In particular, no previous study has phenotypically characterized *Streptococcus* species from Epirus sheep milk for both safety and technological traits. Therefore, in this study, animal streptococci that coexisted with autochthonous enterococci in native Epirus breed sheep milk before or after thermization [24] were biotyped, biochemically evaluated for primary technological and safety properties, and their phenotypic AR was assessed. The final aim was to select candidate strains for inclusion in novel complex NSCs or as adjuncts in future traditional cheese-making studies.

Materials and methods

Sheep milk isolates, reference strains and culture conditions

Sixteen autochthonous *Streptococcus* spp. isolates originating from two batches of sheep milk of native Epirus breeds before (KFM isolates) and after (KTM isolates) thermization (65ºC; 30 s), and previously identified at the species level by 16S ribosomal RNA (rRNA) gene sequencing [23], were selected for further study (Table 1).

Table 1. Animal streptococci from Epirus sheep milk included in this study[1].

Species	Raw milk isolates	Thermized milk isolates	Closest relative strain in BLAST (Accession no.)	16S rRNA gene seq. similarity (%)	Alternative genotypic species identification
Streptococcus parauberis	KFM 32		MT579801	100	
	KFM 33		MT597919	100	
	KFM 54		MT579786	100	
	KFM 41		MN758826	100	
Streptococcus parauberis	KFM 34		CP025420	100	
	KFM 35		CP025420	100	
	KFM 42		CP025420	100	
Streptococcus equinus	KFM 5	KTM 2, KTM 5, KTM 8, KTM 9, KTM 16	MF429207	100	
Streptococcus gallolyticus	KFM 26		CP050959	99.93	*Streptococcus pasteurianus* (MK330581; 99.93%)
Streptococcus lutetiensis	KFM 55		LS483348	100	

Table 1. Animal streptococci from Epirus sheep milk included in this study[1]. (*continued*)

Species	Raw milk isolates	Thermized milk isolates	Closest relative strain in BLAST (Accession no.)	16S rRNA gene seq. similarity (%)	Alternative genotypic species identification
Streptococcus lutetiensis	KFM 60		LS483403	100	*Streptococcus infantarius* (MK330572; 100%)

[1] All data for the RM isolates (coded with the prefix KFM) are adapted from Sioziou et al. [23]. Counterpart TM isolates (coded with the prefix KTM) are reported by Samelis et al. [6].

Two clinical *Enterococcus* strains were used as reference controls in the AR tests: *Enterococcus faecalis* ATCC 29212™ [25], a quality control strain defined by the Clinical and Laboratory Standards Institute (CLSI) [26], and *Enterococcus faecium* 315VR, a virulent, MDR human isolate primarily serving as a vancomycin-resistant control strain [27]. Additionally, two basic NSC strains, *Streptococcus thermophilus* ST1 and the wild, nisin A-producing (NisA+) *Lactococcus lactis* subsp. *cremoris* M78 [27–29] were included in the present biochemical assays, as specified in the Results.

All sheep milk isolates and the control strains were resuscitated by transferring 0.1 mL of frozen (–30°C) working stock cultures with 20% (w/v) glycerol, in 10 mL MRS broth (Neogen Culture Media, Lab M, Heywood, UK), incubated at 30°C for 24 h, and then subcultured twice, as above. Particularly the thermophilic *Streptococcus* isolates of the SESB complex [20] were also resuscitated and subcultured in 10 mL M17 broth (Merck, Darmstadt, Germany), incubated at 37°C for 24 h to ensure their optimal recovery and growth. The second subculture of all isolates was streaked for growth on MRS agar (Neogen) or M17 agar (Biolife, Italiana, S.r.l., Milano, Italy) at 30°C or 37°C for 48–72 h, to check and ensure purity by transferring one single colony to new 10 mL MRS or M17 tubes, incubated as above, for use in the experiments.

Biochemical identification of the sheep milk isolates

All autochthonous sheep milk isolates were rechecked for their Gram-positive and catalase-negative reactions and basic phenotypic traits: microscopic appearance, CO_2 production from glucose, NH_3 production from arginine, growth at 10°C and 45°C, growth in 4% and 6.5% NaCl, and growth on Kanamycin Aesculin Azide (KAA) agar (Neogen). Next, all isolates were tested for acid production from 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 pre-sterilized 96-well mini-plates (Sarstedt AG & Co. KG, Nümbrecht, Germany) to identify them biochemically at the species, in comparison with their 16S rRNA species identification (Table 1). The mini-plates were incubated at 30°C for 72 h or at 37°C for 48 h for the mesophilic and thermophilic isolates, respectively; the positive sugar fermentation readings were based on the color change of the bromocresol purple indicator to yellow due to acid production. The biochemical discrimination and identification of animal *Streptococcus* spp. was based on the sugar fermentation patterns tabulated in Bergey's Manual of Systematic Bacteriology [11].

Additionally, all 16 *Streptococcus* sheep milk isolates (Table 1) were identified by the API 20 STREP biochemical method, according to the manufacturer's instructions (BioMerieux, Marcy l'Etoile, France). This commercialized id-kit detects for 10 key taxonomic sugar fermentation reactions and 10 key enzymatic activities within the genus *Streptococcus* and related coccoid LAB genera, mainly *Enterococcus*; a positive or negative β-hemolysis reaction of each strain is required as an additional external test to generate its 7-digit API 20 STREP identification code. According to Sioziou et al. [23] and Samelis et al. [6], all streptococcal isolates in Table 1 were not β-hemolytic, but were α-hemolytic. Therefore, the hemolysis test was not repeated during this study.

However, in the course of this study, safety evaluation of *Streptococcus* spp. by biochemical tests was extended to biogenic amine (BA) formation, an undesirable trait for the selection of autochthonous starter or adjunct LAB strains. Therefore, all sheep milk isolates were tested for their in vitro ability to produce histamine and tyramine by using the improved BA screening method of Bover-Cid and Holzapfel [30]. BA

production was recorded by the color change of the improved BA broth from yellowish to blue or purple, using *E. faecalis* ATCC 29212™ and *E. faecium* 315VR as tyramine-positive controls, as described by Tsanasidou et al. [27].

Antibiotic susceptibility of the *Streptococcus* sheep milk isolates

All *Streptococcus* sheep milk isolates were tested for susceptibility to eight common antibiotics, according to the CLSI [26] protocol using the Kirby-Bauer disk diffusion method (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. [27]. For comparative purposes, the AR testing of the present isolates was conducted together with all *Enterococcus* spp. strain biotypes co-isolated from the same RM/TM samples using the same agar media and reagents [24]. Briefly, commercial antibiotic disks (BioMaxima SA, Lublin, Poland) were used. Two M17 agar plates were spread-inoculated with 100 μL of fresh (24-h) M17 broth cultures of each tested isolate, the inocula were left to absorb for 5–10 min, and then 4 different antibiotic disks were placed over the cell lawn on each plate. All plates were incubated at 37ºC overnight. On the next morning, the diameter of the inhibition zones formed around each disk, including the diameter (6-mm) of the disk, as specified by CLSI [26], was measured with a micrometer (model D15, Mitutoyo, Kanagawa, Japan). The above process was repeated using a series of new fresh cultures, and the mean of the two independent replicate measurements of AR zone for each disk was calculated. M17 agar was used for the AR tests because streptococci and enterococci grow abundantly on this medium exclusively containing lactose, the primary sugar fermented by LAB in milk and dairy products, instead of glucose. However, the AR of some strains was further tested comparatively on Mueller-Hinton agar (Oxoid CM0337, Oxoid Ltd., Basingstoke, UK) medium [26] to account for potential media effects on the size and clarity of the inhibition zones. The results were interpreted according to the breakpoints recommended by CLSI [26] for streptococci and enterococci. As indicated in Sheep milk isolates, reference strains and culture conditions, the CLSI quality control strain *E. faecalis* ATCC 29212™ (i.e., penicillin-resistant) and the MDR strain *E. faecium* 315VR served as positive controls.

Milk acidification capacity

All *Streptococcus* sheep milk isolates were further tested for their ability to ferment milk in pure culture in vitro compared with the NSC strains *S. thermophilus* ST1 and *Lc. lactis* M78. The acidifying activity rate was evaluated by measuring the pH declines (ΔpH) of inoculated (1% v/v) 10% reconstituted skimmed milk (RSM; Neogen) cultures in triplicate 10-mL tubes for each strain grown at 37°C for 6 h, shifted to 30°C for up to 24 h, and finally to 22°C for an additional 24 h, in simulation of the gradual temperature reductions of fresh curds during traditional cheese processing. 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. During incubation and before each pH measurement, the clotting strength and the uniformity of the curd, if any, were recorded macroscopically. Moreover, to check whether the temperature shifting from 37ºC (simulating the cheese milk curdling/fermentation step) to 22ºC (simulating the cheese ripening step) retarded the acidifying activity of the isolates, particularly the members of the thermophilic SESB complex (Table 1), additional RSM cultures inoculated as above were incubated at the optimal growth temperature for each species (i.e., 30ºC, 48 h for *S. parauberis*; 37ºC, 24 h for the SESB complex), and the final RSM pH values and clotting strength were recorded as above and compared. Finally, it should be noted that screening for the milk acidifying activity of the *Streptococcus* isolates during this study was limited to pH measurements; the titratable acidity for the strains potentially selected as natural starters should be measured in real cheese milks.

Bacteriocin-mediated antilisterial activity

Fresh (37ºC; 24 h) MRS broth cultures of all sheep milk isolates were streaked on M17 and MRS agar plates. Following growth at 37ºC 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 [23]. For the *Streptococcus* spp.

isolates causing inhibition halos, bacteriocin activity was tested further by using well diffusion assays: filter-sterilized MRS culture (37°C; 24 h) cell-free supernatants (CFS) were assayed directly or after adjustment to pH 6.2, heating at 100°C for 5 min, and treatment with 1 mg/mL of proteinase K and trypsin (Sigma Aldrich Chemie GmbH, Steinheim, Germany) at 37°C for 3 to 24 h, as described for enterococci by Sioziou et al. [23].

Enzymatic activity

Finally, the two selected antilisterial strains *S. lutetiensis* KFM 55 and KFM 60 [23] were assayed for 19 constitutive enzymes in their cell biomass using the API ZYM semi-quantitative method, according to the manufacturer instructions (BioMerieux) and in direct comparison with the basic NSC strains *S. thermophilus* ST1 and *Lc.* lactis M78. Selection was made in view of their potential suitability for inclusion in NSCs or use as protective adjunct strains in future cheese making studies.

Results

Biotyping of animal streptococci from sheep milk of native Epirus breeds

The basic phenotypic/biochemical differentiating characteristics of the autochthonous *Streptococcus* sheep milk isolates are presented in Table 2. First, it was noted that the nine thermophilic, arginine-negative *Streptococcus* sheep milk isolates molecularly assigned to the non-QPS species *S. equinus*, *S. gallolyticus*, and *S. lutetiensis* were not salt tolerant because all failed to grow in 6.5% salt (Table 2). Likewise, the mesophilic, arginine-positive *S. parauberis* isolates did not grow in 6.5% salt (biotype I) or grew with delay (biotype 2). Of note, all streptococcal isolates with cells forming long chains were *S. equinus*. This animal-associated species was subdominant in RM1 (KFM 5), but it prevailed in TM1 post-thermally (Table 1). In contrast, consistent with its mesophilic nature (Table 2), *S. parauberis* was not isolated from TM2, despite it prevailed in RM2 [23]. *S. parauberis* RM isolates formed two distinct biotypes, I and II, with the biotype I isolates failing to produce acid from cellobiose and sorbitol (Table 2). Notably, all three biotype II isolates (KFM 34, KFM 35, and KFM 42) matched one strain genotype in BLAST, too (Table 1), while all seven isolates shared the (key) sugar fermentation reactions with the species *S. parauberis* [11]. On the other hand, the basic sugar fermentation profiles of the single KFM 26 isolate from RM1 and both KFM 55 and KFM 60 isolates from RM2 were identical (Table 2), despite their 16S rRNA gene identification as two separate species of the SESB complex, *S. gallolyticus* and *S. lutetiensis*, respectively (Table 1). All differed from the RM/TM group genotyped as *S. equinus* (Table 1) by their inability to ferment cellobiose and melibiose (Table 2).

Table 2. Phenotypic and biochemical characteristics of the Epirus sheep milk isolates.

Species	*Streptococcus parauberis*		*Streptococcus equinus*	*Streptococcus gallolyticus*	*Streptococcus lutetiensis*
Biotype	I	II			
No. isolates[1]	4	3	6	1	2
Cell shape	CP/CSC	CP/CSC	CLC	CP	CP/CSC
NH_3 from arginine	+	+	–	–	–
Growth at:					
10°C	+	+	–	–	–
45°C	–	–	+	+	+
4.0% salt	+	+	+/(+)	–	–/(+)
6.5% salt	–	+d	–	–	–
Growth on KAA agar	–	–	(+)/–	(+)	+
Fermentation of:					
L-Arabinose	–	–	–	–	–
Cellobiose	–	+	–	+	+

Table 2. Phenotypic and biochemical characteristics of the Epirus sheep milk isolates. (*continued*)

Species	*Streptococcus parauberis*		*Streptococcus equinus*	*Streptococcus gallolyticus*	*Streptococcus lutetiensis*
Galactose	+	+	+	+	+
Lactose	+	+	+	+	+
Maltose	+	+	+	+	+
Mannitol	–	–	–	–	–
Melibiose	–	–	–	+	+
Raffinose	–	–	+/–*	+	+
Ribose	+	+	–	–	–
Sorbitol	–	+	–	–	–
Sucrose	+	+	+	+	+
Trehalose	+	+	+/–*	+	+
Xylose	–	–	–	–	–

Symbols: +: all isolates were positive; –: all isolates were negative; (+): weak reaction; ++: strong positive reaction; +d: delayed reaction. CP: cocci in pairs; CSC: cocci in short chains; CLC: cocci in long chains. The asterisk attached to the 'minus' symbol in the *S. equinus* column refers to strain KTM 16 in Table 1 that was the only of the six isolates that showed a negative fermentation reaction with raffinose and trehalose. [1] The number of isolates for each species and biotype correspond with the number of the coded isolates as grouped in Table 1.

Therefore, to discriminate the present sheep milk isolates of the SESB complex at, and within, the species, it was necessary to use additional, more specific biochemical identification tools. For this purpose, all *Streptococcus* isolates were tested by the API 20 STREP method and their 7-digit id-codes generated according to the manufacturer's instructions, were submitted to the Veterinary Laboratory, Food Hygiene Department, Ministry of Agriculture, Athens, for validating their identification using the apiweb™ software; the results are presented in Table 3. Based on the recorded codes, only the *S. gallolyticus* KFM 26 strain genotype underwent an excellent identification; its previous 16S rRNA-based assignment to this species (Table 1) was extended to the subspecies *pasteurianus* [31] by the API 20 STREP method (Table 3). Additionally, a very good identification within the SESB complex as *S. bovis* biotype I/II was recorded for the two thermophilic isolates previously identified as *S. lutetiensis* (KFM 55) and/or *S. lutetiensis/S. infantarius* (KFM 60) by 16S rRNA gene sequencing (Table 1). This result was considered valid because the above two species were extracted and raised as novel species after a double sequential reclassification of the heterogeneous SESB complex [32, 33]. Specifically, the former *S. infantarius* subsp. *coli* [32] was raised to *S. lutetiensis* sp. nov. [33], the latter differing, among others, from *S. infantarius* subsp. *infantarius* in being β-glucosidase and esculin-positive [32]. Therefore, according to their positive esculin (β-glucosidase) hydrolysis reaction (Table 3), both KFM 55 and KFM 60 strains clearly belong to *S. lutetiensis* (formerly *S. bovis* biotype II.1) [33].

Table 3. API 20 STREP-based identification and biotyping of the *Streptococcus* isolates from raw or thermized sheep milk (compared to the genomic identification in Table 1)[1].

Test	Reaction/Enzymes	*Streptococcus parauberis*		*Streptococcus equinus*	*Streptococcus gallolyticus*	*Streptococcus lutetiensis*
Biotype		I (4)	II (3)	(6)	(1)	(2)
VP	Acetoin production	+	+	–	+	+
HIP	Hydrolysis (hipuric acid)	+	+	–	–	–
ESC	β-glucosidase hydrolysis	–	–	–	+	+
PYRA	Pyrolidonyl arylamidase	–	–	–	–	–
αGAL	α-galactosidase	–	–	+	+	+
βGUR	β-glucuronidase	–	–	–	+	–
βGAL	β-galactosidase	–	–	+	+	–
PAL	Alkaline phosphatase	–	+	–	–	–

Table 3. API 20 STREP-based identification and biotyping of the *Streptococcus* isolates from raw or thermized sheep milk (compared to the genomic identification in Table 1)[1]. (*continued*)

Test	Reaction/Enzymes	*Streptococcus parauberis*		*Streptococcus equinus*	*Streptococcus gallolyticus*	*Streptococcus lutetiensis*
LAP	Leucine aminopeptidase	+	+	+	+	+
ADH	Arginine dihydrolase	+	+	–	–	–
RIB	D-ribose (acidification)	+	+	–	–	–
ARA	L-arabinose (acidification)	–	–	–	–	–
MAN	D-mannitol (acidification)	–	–	–	–	–
SOR	D-sorbitol (acidification)	–	+/(+)	–	–	–
LAC	D-lactose (acidification)	+	+	+	+	+
TRE	D-trehalose (acidification)	+	+	+	+	+
INU	Inulin (acidification)	–	+	–/(+)**	–	–
RAF	D-raffinose (acidification)	–	–	+	+	+
AMD	Starch (acidification)	–	–/+*	+	–	+
GLYG	Glycogen (acidification)	–	–	+	–	+
βHEM	β-hemolysis	–	–	–	–	–
API code		3043410	3063630 3063631*	0250453 0250473**	5650450	5240453
API-ID		*S. agalactiae* Acceptable	*S. uberis* Uncertain	*S. pneumoniae* Good/ Very good	*S. gallolyticus pasteurianus* Excellent	*S. equinus* (*S. bovis* I/II) Very good

[1] The number of isolates (in brackets) corresponds with the isolate codes as grouped for each species in Table 1. * Isolate KFM 35 of *S. parauberis*-biotype II was starch-positive; ** Isolates KTM 2, KTM 8 and KTM 9 of *S. equinus* gave a weak positive acidification reaction with inulin.

In contrast, the 16S rRNA identification of the KFM 5 isolate and thereby of the five TM isolates sharing this strain biotype as *S. equinus* (Table 1) was not consistent with their very good or good identification as *Streptococcus pneumoniae* by the API 20 STREP method (Table 3). Likewise, there was a discrepancy between the molecular identification of *S. parauberis* biotypes I and II by the 16S rRNA method using BLAST (Table 1) and their 'acceptable' or 'uncertain' identifications as *Streptococcus agalactiae* and *S. uberis*, respectively, using the API 20 STREP id-database (Table 3).

Lastly, regarding cheese milk safety, none of the non-hemolytic *Streptococcus* spp. sheep milk isolates biotyped in Tables 2 and 3 were found to produce histamine or tyramine in improved BA broth, unlike the two virulent control strains *E. faecalis* ATCC 29212™ and *E. faecium* 315VR, which were confirmed to produce tyramine when tyrosine was added as a precursor in BA broth (data not shown).

Antibiotic resistance of animal streptococci derived from Epirus sheep milk

The phenotypic AR profiles of the 16 *Streptococcus* spp. isolates originating from two sheep milk batches of native Epirus breeds before and after thermization are presented comparatively to the AR profiles of the two *Enterococcus* control strains in Table 4.

As anticipated, the *vanA*+ *E. faecium* 315VR strain was MDR whereas the CLSI quality control *E. faecalis* ATCC 29212™ was resistant to penicillin but partially susceptible to most of the remaining antibiotics tested, including vancomycin (Table 4). Because AR is strain-dependent, every RM/TM isolate found resistant to at least one of the tested antibiotics is tabulated separately; otherwise, the remaining antibiotic susceptible or of intermediate sensitivity isolates within each species are combined by averaging the diameter of their mean inhibition zones.

Table 4. Antibiotic susceptibility of the *Streptococcus* sheep milk isolates.

Strain code (biotype)	Antibiotic tested (μg/disc)							
	AMP 10	**CHL 30**	**CIP 5**	**ERY 15**	**GEN 10**	**PEN10U**	**TET 30**	**VAN 30**
Control strains								
E. faecium 315VR**	**R (6.0)**	I (16.1)	**R (6.0)**	**R (6.0)**	**R (6.0)**	**R (6.0)**	**R (9.4)**	**R (7.6)**
E. faecalis ATCC 29212™	S (20.6)	S (22.0)	I (19.1)	I (16.8)	I (9.4)	**R (12.7)**	I (15.1)	I (16.1)
Sheep milk isolates								
S. parauberis KFM 41 (biotype I)	S (24.0)	S (22.7)	**R (15.3)**	S (25.5)	S (18.2)	S (28.0)	**R (12.9)**	S (20.1)
S. parauberis biotype I (three remaining isolates)	S (26.5 ± 1.2)	S (23.6 ± 3.14)	I (18.9 ± 2.4)	S (23.6 ± 1.9)	S (14.1 ± 0.6)	S (27.5 ± 1.1)	S (27.0 ± 0.4)	S (21.0 ± 1.1)
S. parauberis KFM 34 (biotype II)	S (23.8)	S (23.2)	I (18.8)	S (21.7)	S (12.4)	S (22.7)	**R (14.8)**	S (20.6)
S. parauberis KFM 35 (biotype II)	S (28.6)	S (25.9)	S (24.6)	S (26.8)	S (13.1)	S (26.1)	**R (15.0)**	S (25.3)
S. parauberis KFM 42 (biotype II)	S (24.2)	S (22.4)	**R (15.1)**	S (25.2)	S (17.4)	S (28.5)	**R (14.0)**	S (20.6)
S. equinus KFM 5	S (34.1)	S (27.0)	S (21.6)	S (27.0)	S (18.3)	S (29.7)	**R (13.8)**	S (22.2)
S. equinus KTM 8	S (28.2)	S (23.9)	I (19.3)	S (24.9)	S (13.1)	S (27.6)	**R (17.0)**	S (17.5)
S. equinus KTM 9	S (25.2)	S (25.2)	I (19.0)	S (25.6)	S (12.1)	S (26.4)	**R (18.0)**	S (18.1)
S. equinus (three remaining isolates)	S (27.4 ± 1.0)	S (25.8 ± 1.2)	S (21.0 ± 1.5)	S (26.0 ± 1.4)	S (13.1 ± 2.6)	S (25.5 ± 3.4)	S/I (22.7 ± 5.8)	S (19.1 ± 1.0)
S. gallolyticus KFM 26	S (25.3)	S (27.0)	**R (13.8)**	I (19.4)	S (22.7)	S (26.0)	I (21.1)	S (20.7)
S. lutetiensis KFM 55	S (19.5)	S (24.4)	**R (12.1)**	S (25.0)	S (12.8)	S (25.9)	S (27.8)	S (23.0)
S. lutetiensis KFM 60	S (21.4)	S (25.3)	**R (13.4)**	S (21.2)	S (16.8)	S (29.6)	S (28.7)	S (24.4)

AMP: ampicillin; CHL: chloramphenicol; CIP: ciprofloxacin; ERY: erythromycin; GEN: gentamicin; PEN: penicillin; TET: tetracycline; VAN: vancomycin. The numbers next to the antibiotics indicate μg per disk, or units per disk for penicillin. S (susceptible), I (intermediate), **R (resistant**); the numbers in brackets indicate the inhibition zone size (mm) as the mean of two independent measurements for each strain versus each antibiotic disk; SD values (n = 2) for the AR zones of each strain singly are not shown for table simplification.

Contrary to our anticipation, all *Streptococcus* isolates from sheep milk before or after thermization had restricted ARs (Table 4). Specifically, the RM2 *S. parauberis* biotype I isolates were susceptible to all antibiotics tested, except for KFM 41 that was resistant to ciprofloxacin and tetracycline, while all RM2 biotype II isolates were resistant only to tetracycline, and one of them (KFM 42) to ciprofloxacin similarly to KFM 41 above. *S. equinus* KFM 5 from RM1 was strongly resistant to tetracycline, too. However, from the five *S. equinus* isolates from TM1, two were close to the CLSI breakpoint (≤ 18 mm) and another two were of intermediate sensitivity to tetracycline (Table 4). None of the six *S. equinus* isolates were resistant to ciprofloxacin, but it is worthy of noting that the two tetracycline-resistant isolates (KTM 8, KTM 9) from TM1 were of intermediate sensitivity to ciprofloxacin as well. Conversely, *S. gallolyticus* subsp. *pasteurianus* and *S. lutetiensis* strain biotypes represented by the raw sheep milk isolates KFM 26 and KFM 55 = KFM 60, respectively, were fully resistant to ciprofloxacin, but susceptible or partially susceptible (i.e., KFM 26) to tetracycline and the remaining six antibiotics tested. None of the 16 *Streptococcus* sheep milk isolates of the *S. uberis* group or the SESB complex exhibited multidrug resistance (i.e., resistance to ≥ 3 antibiotic classes). Instead, all showed a remarkably high susceptibility to ampicillin, chloramphenicol, erythromycin, gentamycin, and mainly penicillin and vancomycin (Table 4).

The above AR profiles of the *Streptococcus* spp. isolates were confirmed for total eight representative isolates resistant to tetracycline and/or ciprofloxacin when tested on M17 agar plates (Table 4), which were also tested on Mueller-Hinton agar, the reference CLSI agar for disk diffusion AR testing [26]; the results are summarized in Table S1. Generally, the antibiotic inhibition zones on Mueller-Hinton agar (containing no glucose or lactose, but starch plus beef dehydrated infusion and casein hydrolysate) were similar, in some cases smaller, than those formed by the same strain on M17 agar; however, the AR profile of each representative strain was not critically altered. Most discrepancies were minor and noted with ciplofloxacin (Table S1). In fact, the most prominent and thus critical difference was the resistance of *S.*

equinus KFM 5 to ciprofloxacin on Mueller-Hinton agar, whereas it was sensitive on the respective M17 agar plates (Table 4). To address this discrepancy, the AR testing on M17 agar was repeated particularly for this strain and confirmed that ciprofloxacin caused a large (22.9 mm) inhibitory zone on its cell lawn as all antibiotics (> 21 mm) did, except for tetracycline (14.8 mm) to which *S. equinus* KFM 5 was resistant on both agar media. Further tests are needed to clarify this medium-dependent AR discrepancy.

Growth temperature-dependent variations in the milk acidification capacity of the *Streptococcus* sheep milk isolates

When inoculated (1% v/v) in heat-sterilized (121°C; 5 min) RSM (initial pH 6.78) and grown at 30°C for 48 h, the mesophilic *S. parauberis* biotype I and II isolates (Table 2) reduced the pH to 4.99 ± 0.02 (ΔpH 1.79) and 5.09 ± 0.04 (ΔpH 1.69), respectively; this slightly higher milk acidifying capacity of the biotype I isolates resulted in a firmer curd compared to the biotype II isolates which gave a softer unstable curd that was flowed by inverting the tube. It is worth noting that the RSM cultures of all *S. parauberis* isolates remained non-clotted (pH > 5.2) after the first 24 h, requiring at least a 42-h incubation period at 30°C to curdle the milk. Conversely, the six thermophilic *S. equinus* isolates (Table 2) were the best acidifiers among the sheep milk streptococci in this study since their RSM culture pH fell to 4.80 ± 0.02 (ΔpH 1.98) and the skimmed milk was curdled firmly at 37°C for 24 h. Both *S. lutetiensis* KFM 55 and KFM 60 strains were relatively less aciduric (pH 4.89 ± 0.09; ΔpH 1.89) and formed a softer curd than the *S. equinus* isolates in RSM. *S. gallolyticus* KFM 26 was the least acidifying strain (pH 5.34 ± 0.08; ΔpH 1.44) following growth in RSM at 37°C for 24 h (data not tabulated).

From the above findings, it became evident that the gradual acidifying activity of the *Streptococcus* sheep milk isolates was primarily dependent on the species, less so on each strain within a species, and strongly on the growth temperature, as indicated by the kinetic acidification results presented separately for each isolate in Table 5. Indeed, the *S. equinus* isolates were the strongest, and the *S. parauberis* isolates were the weakest RSM acidifiers during the first 6 h at 37°C, with the *S. lutetiensis* and *S. gallolyticus* isolates being of intermediate rapid acidification capacity. Surprisingly, based on the second-term ΔpH values during incubation, retardation of the acidifying activity of all *S. parauberis* isolates was similar (0.16 to 0.29 pH units) with that of the thermophilic SESB isolates (0.16 to 0.27 pH units) following shifting of the incubation temperature from 30°C to 22°C after the first 24 h (Table 5). Therefore, all *S. parauberis* failed to reduce the final RSM pH < 5.2 and curdle the milk when growth was completed at 22°C, instead of 30°C and above. Neither of the two *S. lutetiensis* strains curdled the RSM under the shifting incubation temperature conditions (Table 5), as both did so slowly at 37°C for 24 h. Only *S. equinus* isolates managed firm RSM curds at 22°C thanks to their superior milk acidification rate at 37°C and then at 30°C, while *S. gallolyticus* KFM 26 was the only raw sheep milk strain likely favored by the growth temperature shifting (Table 5).

Table 5. Acidifying activity of the *Streptococcus* spp. isolates from Epirus sheep milk isolates, in comparison with the basic NSC strains, in reconstituted skim milk (RSM) at gradually decreasing incubation temperatures[1].

Species	Isolate code	Shifting incubation (temperature/time) conditions					
		37°C for an initial 6 h		30°C for an additional 18 h		22°C for an additional 24 h	
		RSM pH	Milk curd[2]	RSM pH	Milk curd	RSM pH	Milk curd
Streptococcus parauberis (Biotype I)	KFM 32	6.17	–	5.45	–	5.22	–
	KFM 33	6.20	–	5.47	–	5.29	–
	KFM 54	6.23	–	5.64	–	5.35	–
	KFM 41	6.56	–	5.68	–	5.44	–
Streptococcus parauberis (Biotype II)	KFM 34	6.45	–	5.63	–	5.47	–
	KFM 35	6.50	–	5.63	–	5.43	–
	KFM 42	6.40	–	5.61	–	5.45	–
Streptococcus equinus	KFM 5	5.76	–	4.96	++	4.79	++
	KTM 2	5.86	–	5.21	+	5.05	++

Table 5. Acidifying activity of the *Streptococcus* spp. isolates from Epirus sheep milk isolates, in comparison with the basic NSC strains, in reconstituted skim milk (RSM) at gradually decreasing incubation temperatures[1]. (*continued*)

Species	Isolate code	Shifting incubation (temperature/time) conditions					
		37°C for an initial 6 h		30°C for an additional 18 h		22°C for an additional 24 h	
		RSM pH	Milk curd[2]	RSM pH	Milk curd	RSM pH	Milk curd
	KTM 5	5.82	–	5.19	+	5.02	++
	KTM 8	5.86	–	5.23	+	5.04	++
	KTM 9	5.89	–	5.22	+	5.05	++
	KTM 16	5.90	–	5.24	+	5.03	++
Streptococcus gallolyticus	KFM 26	6.04	–	5.28	(+)	5.01	+
Streptococcus lutetiensis	KFM 55	6.16	–	5.36	–	5.19	–
	KFM 60	6.17	–	5.51	–	5.35	–
Basic NSC strains							
S. thermophilus ST1		4.99	++	4.08	++	4.00	++
Lc. lactis/cremoris M78		5.48	–/(+)	4.66	++	4.46	++

[1] Each pH value is the mean of two RSM replicates; the initial pH of RSM was 6.78. [2] Type of milk curd: ++: hard, strong milk clotting; +: soft, moderate milk clotting; (+): partially and weakly clotted milk; –: no evidence of milk clotting/curd formation.

Meanwhile, the primary starter *S. thermophilus* ST1 grew abundantly in RSM, managing a ΔpH value as high as 1.79 after 6 h at 37°C, which increased further to 2.70 pH units after 24 h at 30°C and resulted in strong curd formation early during incubation. The NisA+ costarter *Lc. lactis* M78 was a remarkably slower milk acidifier than ST1 but faster and stronger than all *Streptococcus* sheep milk isolates (Table 5).

Bacteriocin-mediated antilisterial activity of the *Streptococcus lutetiensis* raw sheep milk strains

All *S. parauberis* sheep milk isolates and mainly *S. gallolyticus* KFM 26 and both *S. lutetiensis* KFM 55 and KFM 60 strains inhibited growth of *L. monocytogenes* no. 10 on the MRS agar (initial pH 5.7 ± 0.1) overlays (i.e., all six *S. equinus* isolates were unable to grow in/on MRS media and thus could not be tested accordingly). However, none of the present *S. parauberis*, *S. equinus*, and *S. gallolyticus* strains could inhibit *Listeria* growth in the respective M17 agar (initial pH 7.2 ± 0.2; containing 0.5% lactose instead of 2% glucose) overlays. As previously noted [23], *S. lutetiensis* KFM 55 and KFM 60 were the only streptococcal raw sheep milk isolates that showed a clear bacteriocin-like antilisterial activity on M17 agar overlays (Figure 1A; the non-active KFM 26 strain is illustrated in Figure 1B for comparison). Next, the direct antilisterial activity of both *S. lutetiensis* strains was retained in their filter-sterilized acidic (pH 4.5–4.7) CFS after growth in MRS broth at 37°C for 24 h (Figure 1C), as well as after the acidic CFS was neutralized at pH 6.2 and boiled at 100°C for 5 min, but it was lost after treatment with proteolytic enzymes, proteinase K and trypsin (Figure 1D). Thus, strains KFM 55 and KFM 60 excreted a heat-resistant antilisterial peptide in MRS broth, most likely a class II bacteriocin. Further studies are needed to find out whether strains KFM 55 and KFM 60 possess one or more known or novel streptococcal bacteriocin gene/s in their genome and whether the active peptide/s can be produced in situ in sheep milk during cheese making or enrichment of the milk with glucose and possibly additional nutrients present in MRS is a prerequisite for bacteriocin formation by *S. lutetiensis.*

Variations in the enzymatic activity profiles of *S. lutetiensis* raw sheep milk strains compared to the basic *S. thermophilus* and *Lc. lactis* cheese starter strains

Based on the AR and biochemical identification results, and particularly on the API 20 STREP records closely relating most strain biotypes of sheep milk streptococci with the pathogenic species *S. agalactiae*, *S. uberis*, *S. pneumoniae*, and *S. gallolyticus* subsp. *pasteurianus* (Tables 2–4), only the two bacteriocin-producing *S. lutetiensis* KFM 55 and KFM 60 strains were further evaluated for their entire enzymatic activity profiles. Major species-dependent variations in the API ZYM profiles of the two *S. lutetiensis* and the two NSC strains, *S. thermophilus* ST1 and *Lc. lactis* M78 were noted (Table 6).

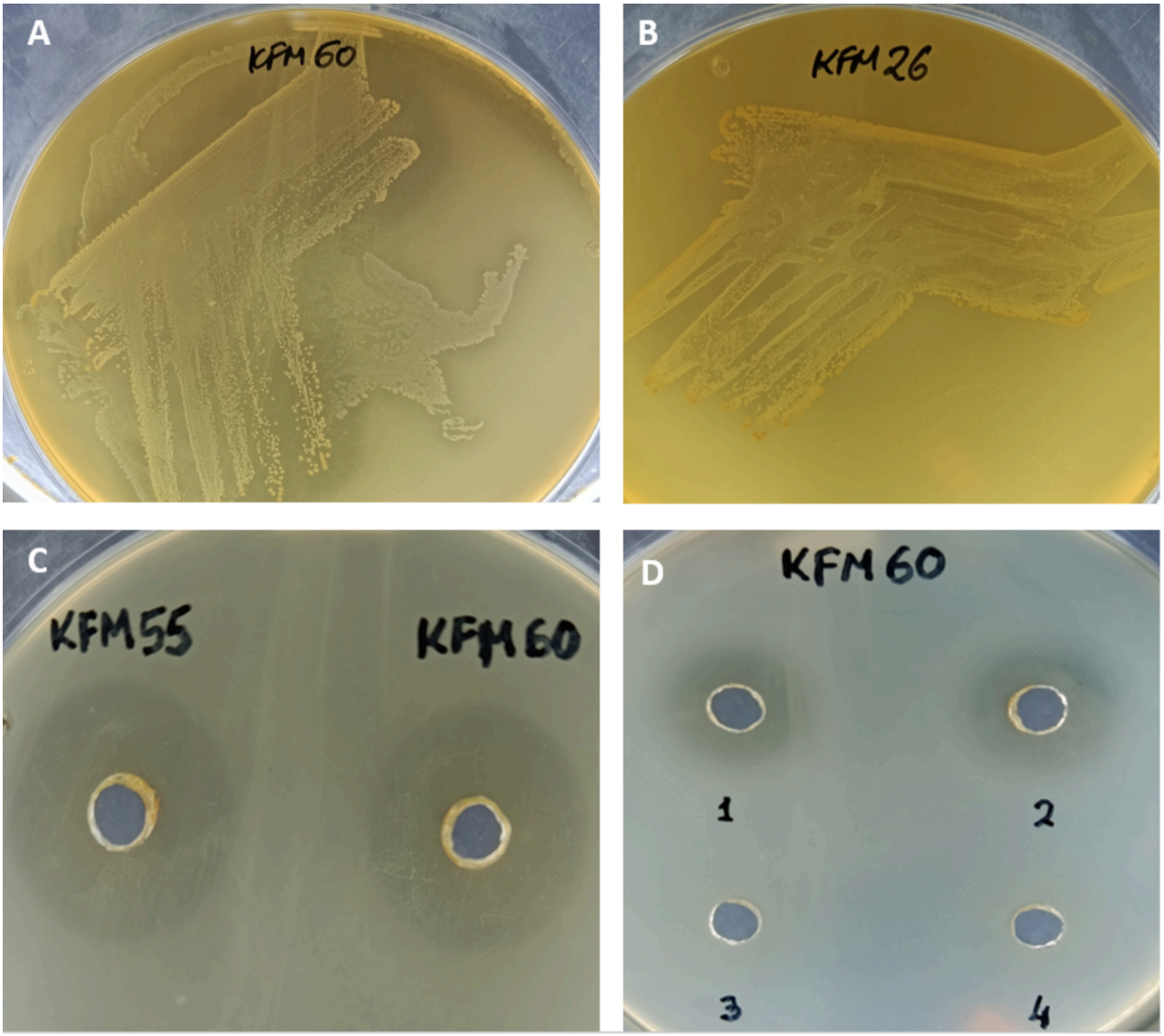

Figure 1. Bacteriocin-mediated activity of the *Streptococcus lutetiensis* KFM 55 and KFM 60 raw sheep milk strains against *Listeria monocytogenes* no.10. (**A**) Direct in vitro antilisterial activity of the streaked KFM 60 strain on M17 agar overlayed with a listerial cell lawn and incubated at 30ºC overnight; (**B**) no activity by *S. gallolyticus* KFM 26; (**C**) listerial growth inhibition by the filter-sterilized MRS (37ºC; 24 h) cell-free supernatants (CFS; pH 4.6–4.7) of strains KFM 55 and KFM 60 in the MPCA well diffusion assays; (**D**) wells opened in a solidified MPCA plate pre-seeded with listerial cells were filled with 80 µL of the neutralized (pH 6.2) CFS of the KFM 60 strain before (well 1) or after (well 2) heating at 100ºC for 5 min, or after treatment with proteinase K (well 3) and trypsin (well 4). KFM 55 gave similar results, not illustrated.

Table 6. Enzymatic activity profiles of selected strains isolated from raw sheep milk of native Epirus breeds with the highest potential for inclusion in natural starter cultures[1].

Strain	*Streptococcus thermophilus* ST1	*Lactococcus lactis* M78	*Streptococcus lutetiensis* KFM 55	*Streptococcus lutetiensis* KFM 60
Alkaline phosphatase	0	40	10	20
Esterase (C4)	20	20	0	0
Esterase lipase (C8)	0	30	0	0
Lipase (C14)	0	0	0	0
Leucine arylamidase	40	40	40	40
Valine arylamidase	10	30	5	5
Cystine arylamidase	10	30	0	0
Trypsin	0	0	0	0
α-chymotrypsin	0	20	0	0
Acid phosphatase	30	40	40	40
Naphthol-AS-BI-	10	40	20	20

Table 6. Enzymatic activity profiles of selected strains isolated from raw sheep milk of native Epirus breeds with the highest potential for inclusion in natural starter cultures[1]. (*continued*)

Strain	*Streptococcus thermophilus* ST1	*Lactococcus lactis* M78	*Streptococcus lutetiensis* KFM 55	*Streptococcus lutetiensis* KFM 60
phosphohydrolase				
α-galactosidase	0	0	40	40
β-galactosidase	30	0	0	0
β-glucuronidase	0	0	0	0
α-glucosidase	0	40	5	0
β-glucosidase	0	0	0	0
N-acetyl-β-glucosaminidase	0	0	0	0
α-mannosidase	0	0	0	0
α-fucosidase	0	0	0	0

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 (similar to the first well-control); 1: liberation of 5 nmol; 2: 10 nmol; 3: 20 nmol; 4: 30 nmol; 5: 40 nmol or more (Samelis and Kakouri [24] 2025). Liberation of 20 to 40 nmol or more was considered a clearly positive enzymatic reaction. The basic starter strains *S. thermophilus* ST1 and *Lc. lactis* M78 were tested for comparison.

Specifically, both *S. lutetiensis* isolates displayed very strong α-galactosidase activity, which was absent in both NSC strains ST1 and M78, which, however, displayed strong β-galactosidase and α-glucosidase activity, respectively (Table 6). Actually, these were the most prominent API ZYM differences between the four strains as far as the crucial glycolytic enzyme system for milk fermentation is concerned. On the other hand, similarly to ST1 and M78, both *S. lutetiensis* strains displayed strong leucine arylamidase and acid phosphatase, and moderate napthol-AS-BI-phosphohydrolase activity. However, unlike M78, both *S. lutetiensis* strains lacked esterase (C4), esterase-lipase (C8), or valine and cystine arylamidase activities, which were also weak or absent in ST1. Moreover, both *S. lutetiensis* strains lacked α-chymotrypsin activity moderately possessed by M78, but not by ST1. Also, although both of them displayed a weak to moderate alkaline phosphatase activity in the API ZYM kit (Table 6), they should be considered negative as they were in the API 20 STREP id-kit (Table 3), which is more reliable for this particular enzymatic test [24]. Finally, no strain showed lipase (C14), trypsin, β-glucuronidase, β-glucosidase, *N*-acetyl-β-glucosaminidase, α-mannosidase, and α-fucosidase activities (Table 6).

Discussion

Except for their phenotypic resistance or intermediate susceptibility to ciprofloxacin (68.8%) and tetracycline (50%), the increased (> 22 mm inhibition zones) sensitivity of all mesophilic or thermophilic animal streptococci from raw or thermized sheep milk to all antibiotics tested (Table 4) was positive for cheese safety. This finding correlates well with the absence of the *gelE*, *hyl*-like or cytolysin genes from all *Streptococcus* RM isolates, primarily from *S. equinus* and the other two thermophilic members of the SESB complex [23], which can survive in TM [6]. It is also consistent with the findings reported by Özkan et al. [20] regarding the beneficial properties of *S. gallolyticus*, *S. lutetiensis* and *S. infantarius* Turkish cheese isolates, including the susceptibility of all, except for one, to ampicillin, chloramphenicol, penicillin, and vancomycin. Likewise, Morandi et al. [34] detected two subdominant *S. equinus* strains in an Italian RM cheese susceptible to all antibiotics (i.e., ciprofloxacin, erythromycin, penicillin, tetracycline, vancomycin) tested herein, too; however, both Italian *S. equinus* strains were resistant to streptomycin, which was not among the antibiotics tested in the present study. Conversely, 43% and 24% of the *S. lutetiensis* isolates from clinical mastitis of dairy cows in China were resistant to tetracycline and erythromycin [16].

Similarly, *S. parauberis* field isolates from clinical bovine mastitis cases in China were highly resistant to erythromycin (90.9%), followed by tetracycline (45.5%), chloramphenicol (36.4%) and clindamycin (27.3%); of note, the *S. uberis* co-isolates were highly resistant to tetracycline (81.3%) and clindamycin (62.5%), while both species were susceptible to ampicillin [15]. Earlier, Pitkälä et al. [35] found subclinical isolates of *S. uberis* from Finish bovine milk to be resistant to oxytetracycline (40.6%) and erythromycin

(15.6%). All of them were susceptible to β-lactam antibiotics, and two subdominant isolates of *S. parauberis* isolates were susceptible to all antibiotics tested [35]. Of note, four *S. uberis* RM cheese strains were susceptible to all antibiotics tested (with the exception of streptomycin), including tetracycline; however, three and one of them were found to possess the *tetM* and *tetL* genes, respectively, despite all of them were phenotypically sensitive to tetracycline [34].

Overall, the high variability in virulence gene profiles of field strains of the two *Streptococcus* groups, *S. uberis/parauberis* [15] and the SESB complex [20] primarily associated with mastitis in ruminants is a serious concern. Additional streptococcal virulence genes (*asa1*, *mrp*, *sly*, *bay*, *bca*, *speG*, *scpB*, *ssa*) were tested for the SESB complex and the species *S. lutetiensis* in particular [16, 20]. Also, the virulence genes (*sua*, *pauA*, *skc*, *gapC*, *hasC*) specific to the pyogenic *S. uberis* group [15] differ from the *Enterococcus*-specific virulence genes tested by Sioziou et al. [23].

Streptococcus-specific virulence genes were not tested by Sioziou et al. [23] or in this study because, as mentioned, according to the API 20 STREP data in Table 3: All *S. parauberis* sheep milk isolates biochemically identified as *S. agalactiae* (biotype I) or *S. uberis* (biotype II), and all *S. equinus* sharing a high biochemical similarity with *S. pneumoniae*, were *a priori* considered unwanted strains resembling closely-related pathogenic species causing mastitis [17, 35–37], irrespective none of them was MDR (Table 4) or β-hemolytic (Table 3) [6, 23]. In other words, the potential inclusion of any of the above non-QPS *Streptococcus* field isolates in complex NSCs is restricted by their genotypic and primary phenotypic assignment to major LAB species associated with clinical mastitis [37, 38], especially to *S. agalactiae* and *S. uberis* with a pooled global prevalence of 9% each amongst the most important pathogens present in the milk of the world [39]. The above restriction also applies to the single *S. gallolyticus* subsp. *pasteurianus* KFM 26 raw sheep milk isolate, the only strain biotype studied herein that exhibited β-glucuronidase activity (Table 3).

In summary, as it was concluded by Özkan et al. [20], dairy scientists should be conscious to claim that strains of non-QPS species within the SESB complex associated with many diseases are actually beneficial. However, when certain safe strains of this major group prevail in traditionally fermented dairy (cheese) products, they deserve to be evaluated differently from similar strains of clinical origin. Amongst the non-QPS sheep milk *Streptococcus* isolates of this study, only the two *S. lutetiensis* strains were likely to deserve further attention and a positive view. The species is highly controversially associated with clinical mastitis of dairy cows [16]. However, different *S. lutetiensis* strains were members of the beneficial technological LAB biota in Turkish Beyaz [40] and Tulum [41] cheeses, or showed high acid and diacetyl/acetoin production in milk after 24 h at 30–40°C [42]. Moreover, unidentified class I and II bacteriocins predicted in the genomes of ruminal *S. lutetiensis* strains were confirmed by SDS-PAGE, showing promise as novel antimicrobial peptides [43]. Therefore, recent data about *S. lutetiensis* are in accordance with our present findings regarding the strong bacteriocin- mediated antilisterial activity (Figure 1), strong acetoin production and beneficial enzymatic activities of the *S. lutetiensis* KFM 55 and KFM 60 strains, including their α-galactosidase and leucine aminopeptidase activities (Tables 3 and 6). However, on the other hand, the resistance of these strains to ciprofloxacin, a fluoroquinolone, is a safety concern, especially for potential human consumption and precludes their free use as natural starter or adjunct culture constituents in traditional cheese technologies.

According to the literature, the most promising, autochthonous starter or adjunct LAB candidates should possess strong to moderate acid phosphatase, aminopeptidase, esterase, esterase/lipase and high glycolytic (α-galactosidase and/or β-galactosidase, or α-glucosidase) activities, and should lack undesired activities, such as β-glucoronidase and β-glucosidase considered to be potential mediators of colon carcinogenesis [44, 45]. However, this negative health-associated consideration has been contradicted in recent reviews: A beneficial, multi-functional role for at least the β-glucosidase activity of many LAB species, especially *Lactiplantibacillus plantarum*, in food fermentation and human health is concluded by Paventi et al. [46], supporting the application of *Lp. plantarum* H25, a strong-positive strain for β-glucosidase activity [27], as an aromatic adjunct in traditional Galotyri PDO cheese products [28]. Nevertheless, neither the two selected bacteriocin-producing *S. lutetiensis* strains nor the two primary NSC

strains *S. thermophilus* ST1 and *Lc. lactis* M78 were β-glucosidase-positive (Table 6), whereas the only β-glucoronidase-positive, potentially harmful sheep milk isolate was assigned to *S. gallolyticus* subsp. *pasteurianus* KFM 26 (Table 3).

On the other hand, the superiority of the cheese starter strain *S. thermophilus* ST1 to strongly acidify the RSM within less than 6 h at 37ºC (Table 5) was primarily attributed to its positive reaction for β-galactosidase, i.e., the main enzyme responsible for lactose breakdown to glucose and galactose during milk fermentation. Accordingly, the faster and stronger RSM acidification by all *S. equinus* isolates and *S. gallolyticus* KFM 26 (Table 5) corroborates their β-galactosidase-positive reaction in the API 20 STREP compared to all *S. parauberis* and *S. lutetiensis* isolates, which gave a negative reaction (Table 3). In particular, the weaker milk acidifying activity of the *S. parauberis* isolates is consistent with the fact that all of them were negative for β-galactosidase, α-galactosidase, β-glucosidase, and β-glucuronidase; their α-glucosidase activity, not included in the API 20 STREP kit (Table 3), should be tested in future studies.

Meanwhile, the lack of α-galactosidase in both NSC strains ST1 and M78 (Table 6), and in all strain biotypes of the *E. faecium/E. durans* group and *E. faecalis* that coexisted in both sheep RM/TM counterparts [24] makes α-galactosidase activity exhibited by *S. lutetiensis* a potentially major biotechnological advantage. Whereas the negative lipolytic and low proteolytic (valine and cystine arylamidase) activities of *S. lutetiensis* strains may be replaced by the bioprotective *Lc. lactis* strain (M78 = M104) genotype [29] and selected *E. faecium/E. durans* adjunct strains [27] with respect to their in situ metabolic performance in cheese milk and dairy foods overall. Thus, the prominent differences in the API ZYM profiles of the *S. lutetiensis* KFM 55 and KFM 60 strains might be complementary to those of the NSC strains *S. thermophilus* ST1 and *Lc. lactis* subsp. *cremoris* M78 (Table 6), as well as of the adjunct strains *E. faecium* KE82 (*entA/entB/entP* producer) [47] and *Lp. plantarum* H25 [28].

To this end and relative to the natural presence of controversial, potentially beneficial *S. lutetiensis* strains in Greek RMs from native small ruminant breeds, a very recent (available online 1 May 2026) comprehensive study by Zoumpopoulou et al. [48] on the LAB ecology of eight RM samples of the Lesvos breed identified two *S. lutetiensis* (1.7%) along with four *S. macedonicus* (3.4%) strains as subdominant members of the total LAB biota (117 isolates) dominated by *Enterococcus* spp. (73; 62.4%) followed by thermophilic lactobacilli (27; 23.1%) and mesophilic lactobacilli of the *Lacticaseibacillus casei/paracasei/rhamnosus* group (11; 9.4%). Of note, two strains of the latter probiotic group possessed bacteriocin-mediated antilisterial activity and two probiotic *S. macedonicus* strains anti-streptococcal activity. Conversely, none of the two *S. lutetiensis* strains originating from one sample of Lesvos breed sheep milk produced bacteriocin; however, one of them, *S. lutetiensis* 307, exhibited more than one probiotic properties, including proteolytic activity, bile salt hydrolase (BSH) activity, high survival in the presence of 1% bile salts, and good survival under GIT (pH 2.5) conditions [48]. Finally, both *S. lutetiensis* strains found in Lesvos raw sheep milk were resistant to gentamicin, kanamycin and streptomycin; but, consistent with the KFM 55 and KFM 60 Epirus milk strains, both were susceptible to ampicillin, chloramphenicol, erythromycin, tetracycline and vancomycin, while resistance to ciprofloxacin was not tested for the LAB isolates from Lesvos breed sheep milk by Zoumpopoulou et al. [48].

Collectively, based on the present results and preceding discussion, further in situ validation studies are required to assess the actual milk acidification activity and other potentially complementary enzymatic activities, as well as potential probiotic activities, of the two novel antilisterial-bacteriocin-producing *S. lutetiensis* KFM 55 and KFM 60 strains from Epirus sheep milk in real cheese fermentation and ripening trials.

Finally, it should be noted that the present study has certain limitations. First, the number of animal streptococci tested was small, originating from two sheep milk batches, which limits the strain variability at or within the species level. These sample size limitations further preclude the present results from being generalized to all native sheep breed milks produced in Epirus or other Greek regions. Second, biotyping supported by 16S rRNA gene identification only was insufficient to fully resolve the species and subspecies heterogeneity within the SESB complex, potentially leading to culture-dependent strain misidentifications.

Culture-independent approaches are considered more suitable and powerful to map the complex microbial (LAB) ecology of sheep milk, particularly when it is raw. Whole-genome sequencing of promising or probably unsafe *Streptococcus* strains is needed.

Conclusions

Despite *S. lutetiensis* is a non-QPS species of the SESB complex that causes mastitis, selected aciduric or antagonistic strains of *S. lutetiensis* from artisanal cheeses lacking streptococcal virulence and AR genes have been proposed as starters or adjuncts. In alignment, the antibiotic susceptible (except ciprofloxacin) *S. lutetiensis* KFM 55 and KFM 60 strain biotypes from RM of native Epirus sheep breeds possess beneficial fermentative and enzymatic activities in vitro and antilisterial bacteriocin activity. The structural, either known or novel, bacteriocin gene/s or other antagonistic mechanism underlying the in vitro bacteriocin-mediated activity against *L. monocytogenes* has yet to be elucidated, along with a complete safety evaluation of these strains. Although both lack β-hemolytic activity and the *vanA*, *vanB*, *gelE*, *hyl* or *cyl* genes and are not MDR, the possibility of possessing other virulence genes associated with the SESB complex and the species *S. lutetiensis* in particular remains. Therefore, until their safety status is fully clarified, the inclusion of the phenotypically ciprofloxacin-resistant *S. lutetiensis* KFM 55 and KFM 60 strains in complex NSCs for commercial use in traditional Greek cheeses is hampered. Whole-genome sequencing and in situ cheese trials are required before recommending these strains for commercial NSC development.

Abbreviations

AR: antibiotic resistant

BA: biogenic amine

CFS: cell-free supernatants

CLSI: Clinical and Laboratory Standards Institute

GRAS: Generally Recognized as Safe

KAA: Kanamycin Aesculin Azide

LAB: lactic acid bacteria

MDR: multidrug resistant

NSCs: natural starter cultures

PDO: Protected Designation of Origin

QPS: Qualified Presumption of Safety

RM: raw milk

rRNA: ribosomal RNA

RSM: reconstituted skimmed milk

SESB: *Streptococcus equinus*/*Streptococcus bovis*

TM: thermized milk

Supplementary materials

The supplementary table for this article is available at: https://www.explorationpub.com/uploads/Article/file/1010187_sup_1.pdf.

Declarations

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Author contributions

JS: Conceptualization, Formal analysis, Methodology, Validation, Resources, Data curation, Project administration, Writing—original draft, Writing—review & editing, Investigation, Visualization. AK: Formal analysis, Validation, Writing—review & editing. Both authors read and approved the submitted version.

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The authors declare that they have no conflicts of interest.

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