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Major ecological shifts within the dominant nonstarter lactic acid bacteria in mature Greek Graviera cheese as affected by the starter culture type

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ABSTRACT

Traditional Greek Graviera cheese is often produced from thermized milk to control undesirable bacterial contaminants. Since thermization also reduces the desirable lactic acid bacteria (LAB) microbiota of raw milk, natural undefined or commercially defined starters are used. This study evaluated effects of the type of starter added to bulk thermized milk on the microbiology of mature (day-90) Graviera cheese. Cheeses produced with a natural starter culture (NSC) in non-concentrated yogurt-like form or a commercial starter culture (CSC) containing *Streptococcus thermophilus* and various *Lactococcus lactis* strains in concentrated freeze-dried form, were analyzed microbiologically, and 200 LAB isolates (100 from each type of cheese) were identified. The LAB microbiota of the mature CSC-cheeses was dominated by nonstarter strains of *Lactobacillus paracasei* and *Lb. plantarum* whereas indigenous *Enterococcus faecium* and *E. durans* strains of high phenotypic and genotypic diversity predominated in the respective NSC-cheeses. Populations of enterococci in CSC-cheeses were subdominant by 10 to 100-fold compared with those in NSC-cheeses; *E. faecium* was the most frequently isolated *Enterococcus* species from the mature CSC-cheeses. Sporadic or no isolates of other LAB species, including the commercial *S. thermophilus* and *Lc. lactis* starter strains in the CSC-cheeses and the natural *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus* starter strains plus indigenous *Lactococcus*, *Leuconostoc* and *E. faecalis* in the NSC-cheeses, were detected. In conclusion, the replacement of the NSC with the CSC controlled growth of dairy enterococci in favor of mesophilic nonstarter lactobacilli during ripening. While safety concerns associated with the inefficiency of NSCs to prevent outgrowth of indigenous enterococci suggest that CSCs should be preferred by traditional Greek Graviera cheese processors, panel sensory evaluations showed that the NSC-ripened cheeses were of slightly lower appearance but of occasionally higher flavor scores than the CSC-ripened cheeses.

1. Introduction

According to one of the most comprehensive reviews on cheese microbiology published at the entry of the 21st century (Beresford et al., 2001), dairy LAB are divided in two major biotechnological groups, the starter LAB (SLAB) and the nonstarter LAB (NSLAB). SLAB are intentionally added or naturally enriched in the milk in order to ferment lactose and, thereby, enhance acidification during early cheese making steps. Conversely NSLAB, also characterized as 'secondary flora', occur naturally in raw milk or derive from other contamination sources in dairy farms or plants and generally grow with delay during cheese ripening (Beresford et al., 2001; Kagkli et al., 2007). Their growth occurs at the expense of milk substrates which are not metabolized by SLAB, or catabolic SLAB byproducts, or intracellular nutrients released from the SLAB cells after autolysis (Gobbetti et al., 2015; Sgarbi et al., 2013). While the SLAB group includes a fistful of species, *Streptococcus thermophilus*, *Lactococcus lactis*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* and few *Leuconostoc* spp., the NSLAB group is far more diversified. The most frequent and technologically important NSLAB in traditional cheese fermentations are the genomic groups of mesophilic, facultative heterofermentative *Lactobacillus casei/paracasei/rhamnosus* and *Lb. plantarum/paraplantarum/pentosus*, several obligatory heterofermentative *Leuconostoc*, *Weissella* and *Lactobacillus* spp., and *Enterococcus* spp., primarily *E. faecium*, *E. faecalis* and *E. durans* (Beresford et al., 2001; Gobbetti et al., 2015; Montel et al., 2014). The decisive, multi-functional role of NSLAB group to improve cheese quality and provide health benefits, with special emphasis on the pros and cons for using mesophilic NSLAB lactobacilli as secondary/

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adjunct starters for cheese ripening, were critically updated in recent reviews (Gobbetti et al., 2015; Settanni and Moschetti, 2010). The functional controversial role, probiotic traits and safety concerns associated with the application of enterococci in foods have also been reviewed extensively (Franz et al., 2011; Giraffa, 2003; Moreno et al., 2006; Ogier and Serror, 2008). Harmless *Enterococcus* strains have been applied as secondary adjunct or protective cultures in factory-scale Italian PDO Pecorino and Greek Graviera cheese productions during the last decade (Giannou et al., 2009a; Guarcello et al., 2016; Settanni and Moschetti, 2010). Overall, the most recent and efficient applications of selected *Enterococcus* strains in all types of dairy products from 2000 to date have been tabulated and reviewed by Silva et al. (2018).

Graviera is the finest and most popular traditional Greek cooked hard cheese (Giannou et al., 2009a; Litopoulou-Tzanetaki and Tzanetakis, 2011). Three varieties, Graviera Kritis (Crete), Naxou and Agrafon, have PDO recognition (Anonymous, 2014; Bozoudi et al., 2016). Most Graviera varieties are made of ewe's milk mixed with up to 30% goat milk. The milk may be processed raw, thermized or pasteurized without or with addition of natural (NSC) or commercial (CSC) starter cultures (Kandarakis et al., 1998; Litopoulou-Tzanetaki and Tzanetakis, 2011; Samelis et al., 2009a), in general accordance with the type of cheese milk – type of starter culture occasions categorized by Settanni and Moschetti (2010). Commercial factory-scale Graviera cheese research studies were first conducted in a small, semi-industrial Greek plant (Pappas Bros. Traditional Dairy, Epirus) in the course of the FP6-TRUEFOOD 2006–2010 project (www.truefood.eu); all cheeses were produced from thermized ewes'/goats' milk supplemented with either craft-made, undefined, non-concentrated yogurt-like NSCs or concentrated, freeze-dried CSCs for direct vat set (DVS) application which were imported from Italy and composed of mixed-LAB strains of natural origin and unexposed to any genetic alteration (Samelis et al., 2009b, 2010). Graviera cheese trials fermented with the CSCs showed typical SLAB/NSLAB growth patterns and species successions from fermentation to ripening (Beresford et al., 2001 and Settanni and Moschetti, 2010). Also CSC-cheeses ripened within six weeks after manufacture (Samelis et al., 2010, 2011) whereas the NSC-cheese trials underwent a relatively slower fermentation and their technological microbiota was dominated by indigenous enterococci (unpublished data). Because the predominance of *Enterococcus* spp. in ready-to-eat (RTE), NSC-ripened Graviera cheeses raised safety concerns (Giannou et al., 2009b), published studies on the biodiversity and behavior of *L. monocytogenes* in the CSC-ripened Graviera cheeses were prioritized (Giannou et al., 2009a; Samelis et al., 2009b, 2010, 2011). Concurrently, several NSC-ripened Graviera trials and other types of artisan Greek cheeses served as 'natural reservoirs' for building up a collection of autochthonous SLAB and NSLAB (> 1500 total isolates) in the microbiology laboratory of Dairy Research Institute (DRI, Ioannina).

Recent research aimed at fulfilling biochemical and molecular characterizations of selected indigenous LAB isolates for use as novel starter or adjunct cultures, with emphasis on bacteriocinogenic strains and their factory-scale applications in cooked hard cheese production (Noutsopoulos et al., 2017; Vandera et al., 2017). In specific, this polyphasic identification study reports on the occurrence of major quantitative and qualitative shifts in the NSLAB microbiota predominating in RTE Graviera cheeses after three months of full maturation (Anonymous, 2014), as affected by the SLAB culture type (NSC or CSC) added to the thermized cheese milk. The most pronounced differences in NSLAB diversity are critically discussed.

2. Materials and methods

2.1. Cheese production and sampling

Eight Graviera cheese batches produced with either NSC (4 batches) or CSC (4 batches) were studied. All batches were manufactured from thermized ewes'/goats' (90:10) milk under the same protocol, except for the SLAB type empirically associated with the milk thermization temperature. Specifically, each bulk of milk (2000 L) was used for the production of two counterpart cheese batch trials, one with NSC in milk thermized at 60 °C for 30 s (Giannou et al., 2009b) and the other with CSC in milk thermized at 63 °C for 30 s (Giannou et al., 2009a). The NSC was a craft-made, non-concentrated fresh yogurt-like mixture of *S. thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus;* its composition was analyzed in Section 2.3. The CSC was a freeze-dried concentrate powder mixture of *S. thermophilus*, *Lactococcus lactis* subsp. *lactis*, *Lc. lactis* subsp. *lactis* var. *diacetylactis* and *Leuconostoc* strains for DVS application in the milk (GR02, Mofin Alce Group, Novara, Italy). Following addition of the NSC (5 kg/1000 L) or the CSC (50 U/1000 L) and rennet (40 g/1000 L; Natural rennet powder Tsakanikas, Ioannina, Greece) in cooled (32–34 °C) bulk milk after thermization, cheese processing and ripening were carried out under standard in-plant operations, as described previously (Samelis et al., 2009b, 2010). After 90 days from cheese manufacture, two mature cheeses from each batch were shipped to the DRI microbiology laboratory at Ioannina for analyses.

2.2. Cheese analyses

For microbiological analyses, samples on the mature Graviera cheeses (*ca.* 14 kg each) were collected with presterilized 1-cm-diameter cork borers. Each sample (25 g) was from duplicate cores taken from one cheese. Cheese samples were mixed with 225 mL of sterile 0.1% (w/v) buffer peptone water (BPW, Lab M, Heywood, UK) and stomached (Lab Blender, Seward 400, London, UK) for 1 min at room temperature. For each sample, appropriate decimal dilutions in BPW were prepared, and 0.1 mL or 1 mL was spread or poured, respectively, in duplicate on/in agar plates. All samples were analyzed for the direct enumeration of main microbial groups using the microbiological agar media and incubation conditions tabulated by Samelis et al. (2009a). The presence of natural *Salmonella* and *Listeria* spp./*L. monocytogenes* contamination was detected by culture enrichment in 25 g each of the two cheeses tested per batch, using the microbiological media and pathogen identification kits reported previously (Giannou et al., 2009a; Samelis et al., 2009a).

For the purposes of this study, grated samples of mature (day-90) Graviera cheeses were analyzed for pH and their moisture and salt contents only, as described previously (Samelis et al., 2010). Furthermore, all fully-ripened RTE cheese batches were subjected to comparative sensory panel evaluations. Their attributes (appearance, max. score 10; texture, 40; flavor, 50; total quality, 100) were evaluated by a five-member trained sensory panel as advised by the IDF (1987), and was performed recently for several CSC-mediated Graviera cheese trials ripened under either continuous or sequential air ventilation in the Pappas Bros. (Skarfi E.P.E.) industrial ripening room advanced with the Smart-Ripe prototype (Corrieu et al., 2018).

2.3. Isolation of the predominant LAB microbiota from the mature Graviera cheeses

To comparatively determine the composition of the technological LAB microbiota in NSC-ripened and CSC-ripened cheeses, a constant isolation procedure was used (Samelis et al., 2010). After enumeration, 200 colonies were isolated to represent the predominant Graviera cheese microbiota grown on the first five LAB-supportive agar media (40 isolates per medium) indicated in Table 1. Specifically, five colonies were randomly picked from one highest dilution plate containing 25–100 colonies per agar enumeration medium and incubation temperature conditions. In this manner, 100 presumptive LAB isolates were obtained for each type (NSC or CSC) of fully ripened Graviera cheeses. Before that the NSC and CSC starters were analyzed to confirm their LAB species constituents on the day of cheese manufacture. The fresh yogurt-like NSC was handled like a soft acid-curd cheese sample subjected to all analyses reported above, while the freeze-dried CSC

Table 1
Populations of different groups of lactic acid bacteria (LAB) in mature (day-90) Greek Graviera cheese manufactured from thermized milk with addition of either a natural starter culture (NSC) or a commercial starter culture (CSC).

Starter culture type	Milk thermization	Population (log CFU/g) on total and LAB-selective agar media[a]					
		Total mesophiles (TSAYE; 30 °C, 72 h)[b]	Mesophilic LAB (M-17; 22 °C, 72 h)	Mesophilic & thermophilic LAB (M-17; 42 °C, 48 h)	Mesophilic LAB (MRS; 30 °C, 72 h)	Thermophilic LAB (MRS; 45 °C, 48 h)	Enterococci (KAA; 37 °C, 48 h)
NSC	60 °C, 30 s	8.67 ± 0.26 a A	8.07 ± 0.05 a B	7.83 ± 0.25 a B	8.52 ± 0.41 a A	8.01 ± 0.26 a AB	7.76 ± 0.20 a B
CSC	63 °C, 30 s	8.20 ± 0.35 a A	8.04 ± 0.30 a A	8.02 ± 0.48 a A	8.31 ± 0.36 a A	7.08 ± 0.59 b B	6.97 ± 0.42 b B

[a] Values are the mean ± standard deviation from four cheese batches for each starter culture type. Within a row, means with different capital letters are significantly different ($p < 0.05$). Within a column, means with different lowercase letters are significantly different ($p < 0.05$).
[b] TSAYE, tryptic soy agar plus 0.6% yeast extract; MRS, de Man Rogosa Sharpe agar; KAA, kanamycin aesculin azide agar.

powder was analyzed as recently described by Vandera et al. (2017).

2.4. Biochemical characterization and screening of cheese isolates for antagonistic activity

All 200 isolates were cultured, purified, maintained, and identified biochemically as described by Samelis et al. (2009a, 2010). Only the LAB isolates (gram-positive, catalase-negative, non-sporogenic cocci or bacilli) were further tested for gas production from glucose, arginine hydrolysis, growth at 15 °C and 45 °C, in 2%, 4% and 6.5% (w/v) salt, on kanamycin esculin azide (KAA) agar, and for the fermentation of 13 differentiating sugars selected: L-arabinose, cellobiose, galactose, lactose, maltose, mannitol, melibiose, raffinose, ribose, sorbitol, sucrose, trehalose and xylose (Sigma-Aldrich Chemie GmbH, Steinheim, Germany); fermentation tests were done in sterile 96-well mini-plates. Presumptive enterococcal isolates that showed abundant growth on KAA agar were confirmed for growth at pH 9.6 and survival at 60 °C for 30 min (Samelis et al., 2009a). Afterwards, all LAB isolates were screened for antilisterial activity by a rapid agar overlay technique (Samelis et al., 2010). Briefly, freshly streaked colonies of the isolates on M17 agar plates were covered with a thin 10-mL layer of melted (45 °C) Brain Heart Infusion (BHI) agar (Lab M) seeded (0.2% v/v) with an overnight culture of *L. monocytogenes* N-7143, a sensitive indicator strain to several LAB bacteriocins (Samelis et al., 2009a; Vandera et al., 2017). The LAB isolates surrounded by a well-defined (> 2 mm) clearness of the *Listeria* agar overlay lawn after overnight incubation at 30 °C were further tested for antilisterial activity of their cell-free MRS culture supernatants by a well diffusion assay against seven *L. monocytogenes* indicator strains, including the clinical reference strain Scott A and the non-virulent reference strain no.10 (Samelis et al., 2009a; Vandera et al., 2017).

2.5. Biochemical grouping and bio-typing of cheese isolates - selection criteria at the strain level

Based on the responses of all isolates to the above biochemical tests, distinct 'biotype' groups within each LAB species were formed. All isolates with identical biochemical traits were assumed to represent multiple colony isolations of one biotype. Hence the number of biotypes was considered as the minimum number of 'strains' detected within each LAB species based on the identification traits used in this study, *e.g.*, the actual number of strains might increase further upon testing for additional traits. In case two LAB isolates were identical biochemically but differed in their *in vitro* antilisterial activity tests (only one was active), they were regarded as different strains. For all homogenous biochemical LAB groups, which were presumed to comprise single-strain biotypes, random isolates analogically to the group size or importance, were selected for molecular study. Particularly for the *Enterococcus* isolates, which displayed the highest intra-species heterogeneity (see Results), their separate strain status was evaluated by SDS-PAGE analysis of their whole-cell protein profiles, according to Parapouli et al. (2013). Technically the above bio-typing procedure was the most reliable approach for reducing the number of representative (45 out of totally 200) isolates subjected to molecular identification studies including the complete nucleotide sequence of the 16S rRNA gene for which, unfortunately, limited funds were available in our laboratories.

2.6. Molecular identification of representative Graviera cheese LAB isolates

Molecular identification of 45 representative (22.5% of total) LAB isolates was performed by combining several genotypic methods, as appropriate. Genomic DNA used as template for IGS profiling was extracted according to Pu et al. (2002), whereas for all *Lactobacillus* isolates, genomic DNA extraction was performed according to De et al. (2010) and William et al. (2012) v.3. IGS profiling was performed using primer pair R16-1/R23-2R (Nakagawa et al., 1994). The heterogeneity of 16S rRNA gene sequences of the lactococcal species was studied using primers LacF, CreF, and LacreR (Table S1) (Parapouli et al., 2013; Pu et al., 2002). Specific primers (Table S1) based on the polymorphism of the V1 region of 16S rRNA gene sequences of the *Lb. casei/paracasei/rhamnosus/zeae* group strains allowed their identification, according to the method of Desai et al. (2006) and Ward and Timmins (1999). The heterogeneity of the *recA* gene of *Lb. plantarum/paraplantarum/pentosus* group strains allowed their discrimination using specific primers (Table S1) with a multiplex-PCR assay as described by Parente et al. (2010) and Torriani et al. (2001). Randomly Amplified Polymorphic DNA (RAPD) typing was performed with primer M13, as reported by Rossetti and Giraffa (2005). Amplification reactions were conducted in a PTC-100 (version 7.0) thermocycler (MJ Research Inc.) using the Kapa Hifi PCR System (Kapabiosystems, Massachusetts, USA) with an annealing temperature of 60 °C and specific primers (Table S1) for each amplification. The enterococcal 16S rRNA gene amplification products, obtained with primer pair 16SF (9–27 position of *E. coli*) and 16SR (1525–1542 position of *E. coli*) (Karabika et al., 2009) and a Phusion High Fildelity DNA polymerase system (Finnzyme, Finland), were purified using NucleoSpin Extract 2 in 1 (Macherey-Nagel, Germany). Molecular cloning was performed using a Zero Blunt Kit (Invitrogen) according to the manufacturer's recommendations. *E. coli* strain DH5a grown aerobically in LB media at 37 °C was used as the recombinant plasmid host (Hanahan, 1983). Restriction enzyme digestions, ligations, and agarose gel electrophoresis were carried out using standard methodology (Sambrook and Russell, 2001). Cloned fragments were sequenced by CeMIA (Larissa, Greece). Taxonomic analysis was performed by using the GenBank BLAST program (Altschul et al., 1997) at the NCBI website. Nucleotide sequences from related taxa were retrieved from databases and aligned using CLUSTALW (Thompson et al., 1994). Phylogenetic tree and molecular evolutionary analyses were conducted using MEGA version 4 (Tamura et al., 2007).

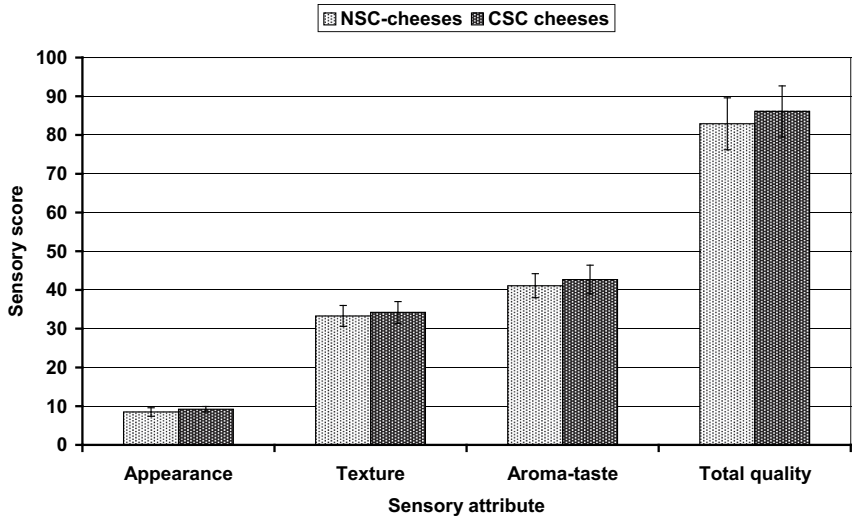

Fig. 1. Panel sensory evaluation based on three attributes and total quality of the mature (day-90) Graviera cheeses manufactured with natural starter culture (NSC; $n = 4$) or commercial starter culture (CSC; $n = 4$).

2.7. Statistical analysis

The microbial quantification data of the NSC-ripened and the CSC-ripened cheese samples were converted to log CFU/g and compared statistically to detect any significant starter type/thermization combined treatment effects on the different microbial populations. For each cheese type, counts on the five LAB isolation agar media were also compared to determine medium/incubation temperature effects. Data were subjected to one-way analysis of variance using Statgraphics (Statistical Graphics Corp., Rockville, MD, USA). Means and standard deviations were calculated, and, when F-values were significant at the $p < 0.05$ level, mean differences were separated by the Least Significant Difference procedure.

3. Results

3.1. Main physicochemical and sensory quality attributes of the NSC-ripened and CSC-ripened cheeses

Mature Graviera cheeses produced with the NSC contained 35.4 ± 0.8% moisture and 1.8 ± 0.3% salt, and had a pH of 5.7 ± 0.2, whereas the corresponding cheeses produced with the CSC had slightly lower pH (5.6 ± 0.1) and moisture content (34.0 ± 1.0%), and slightly higher salt content (1.9 ± 0.3%), respectively.

The panel scores of the mature (day-90) Graviera cheeses for appearance, body/texture, aroma/taste and total quality are summarized in Fig. 1. In general, the CSC-cheeses received higher mean scores than the NSC-cheeses in all attributes, mainly in appearance; however, differences were not significant when the scores of all NSC-cheese batches were combined and compared statistically with the corresponding scores of all CSC-cheese batches (Fig. 1). Batch-to-batch, variations in one or more sensory attributes occurred though, and thus, panel scores often ranged considerably even within the same cheese type. In specific, the mean individual batch score ranges for appearance, texture, aroma/taste and total quality of the NSC-cheeses were 6.9–9.4, 30.8–36.6, 37.0–44.0 and 74.7–89.0, respectively, whereas those of the CSC-cheeses were 8.2–10.0, 32.5–38.4, 38.5–47.0 and 81.0–95.4, respectively. Sensory score ranges were affected by the starter (NSC or CSC) type in the cheeses made of the same bulk milk, but also by other factors associated with seasonal effects on raw milk composition and quality across the milking period (Corrieu et al., 2018); such effects were out of the scope of this study. In summary, while the cheese appearance always improved by application of the CSC, sometimes the NSC-cheeses received slightly higher scores for texture (batch C), aroma/taste (batch B) or total quality (batch C) than their counterpart CSC-cheeses (data not shown). Additionally, according to the critical post-panel discussions between the panelists, the NSC-cheeses were considered to possess more 'artisan-like' flavorings and 'rough' body/texture than the CSC-cheeses which in turn were 'mildly-flavored' and 'industrialized'; thus the two cheese types could be distinguished by the panelists despite they might have received similarly high mean sensory scores for different reasons (Fig. 1).

3.2. Microbiological quality and safety attributes of the NSC-ripened and CSC-ripened cheeses

All cheeses were safe with regard to the pathogens specified in Regulation (EC) 1441/2007 since they contained < 100 CFU/g of coagulase-positive staphylococci while *Salmonella* and *Listeria* were absent in 25-g of cheese. NSC-cheeses had higher ($p < 0.05$) populations of total enterobacteria (2.5 ± 1.0 log CFU/g) than CSC-cheeses (< 1.0 log CFU/g). However, coliform bacteria were undetectable and pseudomonad-like bacteria were < 2.0 log CFU/g in all cheeses, irrespective of milk thermization temperature and starter type. Populations of total ripening (non-pathogenic) staphylococci were also higher ($p < 0.05$) in the NSC-cheeses (5.3 ± 0.9 log CFU/g) than the CSC-cheeses (4.0 ± 0.4 log CFU/g). As regards the predominant technological LAB microbiota, the populations enumerated on the six different agar media under either mesophilic or thermophilic incubation conditions are summarized in Table 1. The only major ($p < 0.05$) difference in the LAB population levels was observed on MRS agar at 45 °C, which coincided with about 10 to 100-fold higher ($p < 0.05$) levels of *Enterococcus* populations in the NSC-cheeses than in the CSC-cheeses (Table 1).

3.3. Biochemical grouping, characterization, and distribution of the Graviera cheese LAB isolates

All isolates were LAB differentiated biochemically in eight LAB species commonly found in milk and cheese (Table 2). The isolates (122 in total) assigned to *E. faecium*, *E. durans*, *Lc. lactis* subsp. *lactis* and *Leuc. mesenteroides* formed heterogeneous groups and several of them were atypical in certain biochemical traits. In contrast, the fewer isolates (28 in total) assigned to *E. faecalis*, *Lb. plantarum* and *S. thermophilus* formed homogeneous groups, while the remaining 50 LAB isolates identified as

Table 2
Biochemical identification and grouping at the species level of 200 lactic acid bacteria (LAB) isolates from mature (90-day-old) traditional Greek Graviera cheeses manufactured with a natural starter culture (NSC; 100 isolates) or a commercial starter culture (CSC;100 isolates).

Species	*Enterococcus faecium*		*Enterococcus durans*		*Enterococcus faecalis*	*Lactobacillus paracasei*		*Lactobacillus plantarum*	*Lactococcus lactis*	*Streptococcus thermophilus*	*Leuconostoc mesenteroides*
	Typical	Atypical	Typical	Atypical		Biotype I	Biotype II		subsp. *lactis*		group
NSC isolates	35	5	30	9	3	2	2	5	4	0	5
CSC isolates	26	0	5	0	0	42	4	17	3	3	0
Total isolates	61	5	35	9	3	44	6	22	7	3	5
Cell shape	C	C	C	C	C	R	R	SR	C	LC	BC
CO_2/glucose	−	−	−	−	−	−	−	−	−	−	+
NH_3/arginine	+	+	+	+	+	−	−	−	5/7	−	−
Slime/sucrose	−	−	−	−	−	−	−	−	−	−	+
Growth at:											
15 °C	+	+	+	+	+	+	+	+	+	−	+
45 °C	+	+	+	+	+	−	−	−	−	+	2/5
4% salt	+	+	+	+	+	+	+	+	+	−	+
6.5% salt	+	+	+	+	+	+	(+)	+	−	−	+
KAA agar	++	++	++	++	++	−	−	+	−	−	−
Fermentation of:											
L-Arabinose	+	+	−	−	−	−	−	+	−	−	3/5
Cellobiose	+	+	+	+	+	+	+	+	+	−	2/5
Galactose	+	+	+	+	+	+	+	+	+	−	+
Lactose	+	+	+	+	+	+	+	+	+	+	+
Maltose	+	+	+	+	+	+	+	+	+	−	+
Mannitol	+	3/5	−	3/9	+	+	+	+	1/7	−	3/5
Melibiose	51/61	+	20/35	2/9	+	−	−	+	−	−	4/5
Raffinose	−	1/5	−	−	−	−	−	+	−	−	4/5
Ribose	+	+	+	+	+	+	+	+	+	−	2/5
Sorbitol	−	3/5	−	2/9	+	+	−	+	−	−	1/5
Sucrose	24/61	+	−	2/9	+	+	+	+	1/7	+	+
Trehalose	+	3/5	12/35	4/9	+	+	+	+	+	−	4/5
Xylose	−	−	−	1/9	−	−	−	−	2/7	−	4/5
Strain biotypes[a]	4	3	4	7	1	1	1	1	4	1	5

Symbols: +, all isolates were positive; −, all negative; 51/61, 51 out of the 61 isolates were positive; (+) weakly positive. For testing the type of growth on KAA: ++, abundant growth, + moderate growth, − no growth. C, cocci; R, rods; SR, short rods; LC, large cocci; BC, bacillococci or elongated cocci.
[a] Indicates the minimum number of strain biotypes within each species on the basis of their key differentiating sugar profiles detailed in Table 5.

Lb. paracasei formed two distinct homogeneous subgroups, one major (biotype I) and the other minor (biotype II) in accordance with their isolation frequency and ability to ferment sorbitol (Table 2). Overall, based on their biochemical grouping, the 200 LAB isolates represented a minimum of 32 individual 'strain biotypes' recovered from eight batches of fully-ripened (day-90) RTE Greek Graviera cheeses (Table 2).

The numerical distribution of the isolates revealed the existence of major compositional differences in the LAB community between the NSC-ripened and CSC-ripened cheeses. In specific, major shifts in the dominant species identified for each type of Graviera cheese (Table 2) plus additional minor differences between the cheese batches produced with the same starter type (Tables 3 and 4) were found. Considering the absence of thermophilic dairy *Lactobacillus* species amongst the GR02-CSC strain constituents, it was interesting to observe that the indigenous thermophilic LAB populations enumerated on MRS/45 °C were subdominant in all CSC-cheeses (Table 1). In contrast, the corresponding thermophilic LAB populations were amongst the predominant groups in all NSC-cheeses; actually in both cheese types thermophilic LAB were at similar population levels with enterococci selectively enumerated on KAA agar. Therefore, to avoid the subdominant MRS/45 °C LAB isolates collected from the CSC-cheeses to interfere with the percentage (%) distribution of the predominant LAB isolates collected from the other four media, the results were split in two tables. Table 3 shows the percentage (%) distribution in the cheeses of the 160 LAB isolates from the MRS/30 °C, M17/22 °C, M17/42 °C and MPCA/37 °C agar plates (40 isolates/medium × 4 agar media), which represented the predominant cheese LAB counted at the −6 or −7 sample dilution (Table 1). Table 4 includes the remaining 40 LAB isolates of the MRS/45 °C populations, which for the CSC-cheeses were collected from plates that were one or two decimal dilutions lower than the corresponding plates of the NSC-cheeses.

Based on their biochemical identification criteria (Table 2), the 160 LAB isolates included in Table 3 were distributed in the cheeses as follows: *E. faecium* (37.5%) and typical (27.5%) plus atypical (10.0%) *E. durans* isolates predominated in the NSC-cheeses. Conversely, biotype I isolates of *Lb. paracasei* (52.5%) predominated in the CSC-cheeses followed by *Lb. plantarum* (21.25%) and *E. faecium* (13.75%). All other LAB species, including the GR02-mixed starter strains of *S. thermophilus* (3.75%) and *Lc. lactis* (3.75%) in the CSC-cheeses and wild *Lc. lactis* (5.0%) and *E. faecalis* (2.5%) in the NSC-cheeses, were isolated at frequencies ≤6.25% (Table 3). Indigenous *Leuconostoc* were sporadically isolated from the NSC-cheeses (6.25%); all five isolates produced slime from sucrose and shared the key phenotypic characteristics of the *Leuc. mesenteroides* group, however, they differed in their main sugar fermentation profiles and therefore were regarded as five different strain biotypes (Table 2). No *Leuconostoc* spp. were isolated from the CSC-cheeses (Table 3) although an undefined 'leuconostocs' fraction was declared amongst the GR02-CSC starter LAB strain constituents by its manufacturer. Neither *S. thermophilus* (Table 3) nor *Lactobacillus delbrueckii* subsp. *bulgaricus* isolates were recovered from the NSC-fermented and ripened cheeses, although those two symbiotic dairy microorganisms were the only LAB species exclusively isolated from the yogurt-like NSC preparations (pH 4.0 ± 0.1). The population of *Lb. bulgaricus* (8.50 ± 0.27 log CFU/g enumerated on MRS agar at 45 °C) in the NSC samples were higher than those of *S. thermophilus* (8.16 ± 0.26 log CFU/g enumerated on M17 agar at 42 °C), a result raising doubts about the actual freshness of the yogurt-like NSC preparations used. The characterization of 20 random isolates (5 isolates/medium × 4 NSC preparations) showed multiple colony isolations of one typical biotype each of the two species from each of the above agar

Table 3
Distribution in mature (day-90) Greek Graviera cheese of 160 lactic acid bacteria (LAB) isolates representing the predominant LAB populations grown on the highest dilution plates of the M17, MRS, TSAYE and M17 agar media incubated at 22, 30, 37 and 42 °C, according to Table 1.

Species	Cheese batch A		Cheese batch B		Cheese batch C		Cheese batch D		Total isolates per starter culture type	
	NSC[a]	CSC[b]	NSC	CSC	NSC	CSC	NSC	CSC	NSC	CSC
Streptococcus thermophilus	–	–	–	1	–	2	–	–	– (0.0%)	3 (3.75%)
Lactococcus lactis	1	1	2	–	–	2	1	–	4 (5.0%)	3 (3.75%)
Lactobacillus paracasei (Biotype I)	–	12	–	9	–	9	2	12	2 (2.5%)	42 (52.5%)
Lactobacillus paracasei (Biotype II)	–	–	1	2	–	1	1	1	2 (2.5%)	4 (5.0%)
Lactobacillus plantarum	–	7	–	2	5	4	–	4	5 (6.25%)	17 (21.25%)
Leuconostoc mesenteroides	–	–	3	–	1	–	1	–	5 (6.25%)	– (0.0%)
Enterococcus faecium[c]	8	–	5	6	9	2	8	3	30 (37.5%)	11 (13.75%)
Enterococcus durans (Typical isolates)	9	–	8	–	1	–	4	–	22 (27.5%)	– (0.0%)
Enterococcus durans (Atypical isolates)	2	–	1	–	2	–	3	–	8 (10.0%)	– (0.0%)
Enterococcus faecalis	–	–	–	–	2	–	–	–	2 (2.5%)	– (0.0%)
Total isolates	20	20	20	20	20	20	20	20	80	80

[a] Cheese manufactured with addition of the natural starter culture (NSC).
[b] Cheese manufactured with addition of the commercial starter culture (CSC).
[c] Typical *E. faecium* isolates are not presented separately from the few atypical isolates because all fermented the primary key sugar, L-arabinose.

media, respectively. From the sugars listed in Table 2, all NSC isolates of *S. thermophilus* fermented lactose and sucrose while those of *Lb. bulgaricus* fermented lactose only (data not tabulated).

In summary, neither the NSC nor the CSC LAB species constituents were prevalent in mature Graviera cheeses (Table 3). Instead, in the NSC-ripened cheeses, *E. faecium* and *E. durans* biotypes accounted for 75% of the predominant LAB microbiota. In contrast, only two NSC-cheese isolates matched biochemically *E. faecalis* (2.5%), which was not isolated from the CSC-ripened cheeses either (Table 3). In consistency, all 40 thermophilic isolates from the MRS/45 °C agar plates were enterococci identified as *E. faecium* and *E. durans*, whereas again *E. faecalis* was sporadic in the NSC-cheeses and undetectable in the CSC-cheeses (Table 4). Overall, the replacement of the NSC with the CSC, associated with an increase of the milk thermization temperature from 60 to 63 °C, reduced the high prevalence of indigenous *Enterococcus* spp. in favor of *Lb. paracasei* and *Lb. plantarum* in the CSC-ripened cheeses (Table 2). While the reason/s for the absence or low presence of *E. faecalis* in all cheeses required genotypic confirmation, *E. durans* seemed to be more vulnerable than *E. faecium* to the antagonistic growth effects of the CSC during fermentation followed by the antagonism exerted by mesophilic NSLAB lactobacilli during ripening (Tables 2–4).

3.4. Intra-species heterogeneity of the isolates and selection criteria for their polyphasic identification

Table 5 presents analytical data on the number of typical or atypical strain biotypes identified for each LAB species (32 biotypes in total; Table 2), the key differentiating sugar fermentation reaction profiles of each biotype, the total number of LAB isolates assigned to each biotype, the numerical distribution of the isolates of each biotype in the cheeses fermented with the NSC and CSC, respectively, and the number and codes of the selected representative isolates from each biotype subjected to molecular identification.

Starting from enterococci, despite their overall high sugar fermentation heterogeneity, all typical (61 isolates; 92.4%) *E. faecium* (biotypes 1A-1D) fermented L-arabinose but not sorbitol, while three

Table 4
Distribution in mature (day-90) Greek Graviera cheese of 40 thermophilic LAB isolates selectively grown on the MRS agar plates incubated at 45 °C; according to the cheese type, the isolates represented either predominant or subdominant *Enterococcus* populations in the cheeses, according to the LAB quantification data in Table 1.

Species	Cheese batch A		Cheese batch B		Cheese batch C		Cheese batch D		Total isolates per starter culture type	
	NSC[a]	CSC[b]	NSC	CSC	NSC	CSC	NSC	CSC	NSC	CSC
Enterococcus faecium[c]	1	5	2	3	2	2	5	5	10 (50.0%)	15 (75.0%)
Enterococcus durans (Typical isolates)	4	–	3	2	1	3	–	–	8 (40.0%)	5 (25.0%)
Enterococcus durans (Atypical isolates)	–	–	–	–	1	–	–	–	1 (5.0%)	– (0.0%)
Enterococcus faecalis	–	–	–	–	1	–	–	–	1 (5.0%)	– (0.0%)
Total isolates	5	5	5	5	5	5	5	5	20	20

[a] Cheese manufactured with addition of the natural starter culture (NSC).
[b] Cheese manufactured with addition of the commercial starter culture (CSC).
[c] Typical *E. faecium* isolates are not grouped separately from the few atypical isolates because all fermented the primary key sugar, L-arabinose.

typical *E. faecalis* isolates (biotype 3A) fermented these two key sugars *vice versa* (Table 5) in compliance with one of the most reliable biochemical keys for enterococci (Manero and Blanch, 1999). All isolates of the typical biotypes of *E. faecium* (1A-1D) and *E. faecalis* (3A) also fermented mannitol, but not raffinose (Table 5), two additional useful characteristics for their discrimination from *E. durans* and other *Enterococcus* species (Manero and Blanch, 1999). Accordingly, the only five (7.6%) isolates distributed in the atypical *E. faecium* biotypes either failed to ferment mannitol (biotype 1G) or fermented raffinose (biotype 1F) and/or sorbitol (biotypes 1E and 1F). Biotype 1F, in particular, included an *Enterococcus* isolate, KE102, which fermented all differentiating sugars except xylose (Table 5) plus amygdalin, dulcitol, inulin, melezitose, methyl-α-D-mannopyranoside, turanose, tagatose and weakly rhamnose in the API 50CHL identification kit (data not tabulated); however, it did not ferment methyl-α-D-glucopyranoside, a reaction discriminating *E. faecium* (negative) from *E. gallinarum* (positive) (Manero and Blanch, 1999). Therefore, KE102 either was a highly atypical *E. faecium* strain or belonged to another species, most probably *E. raffinosus* or *E. hirae*. On the other hand, 79.5% of the *E. durans* isolates were typical (biotypes 2A-2D) because they did not ferment L-arabinose, mannitol, sorbitol, raffinose xylose plus sucrose. Three mannitol-positive *E. durans*-like isolates were assigned to the atypical biotype 2E represented by strain KE98 (Table 5). KE98 plus another six single isolates of atypical *E. durans* strain biotypes (2F–2K), which fermented sorbitol (KE80, KE116, KE88) or sucrose (KE79, KE94) or xylose (KE78) (Table 5), required confirmation by molecular identification. Also one isolate from each of the atypical *E. faecium* biotypes 1E-1G (KE86, KE102 and KE77) plus a total of 18 representative isolates of typical strain biotypes of *E. faecium* (7), *E. durans* (8) and *E. faecalis* (3) were selected (Table 5). Particularly for *E. faecalis*, all three isolates in biotype 3A were selected to assure their identity and homogeneity in consideration of the low isolation frequency of this important *Enterococcus* species from all mature Graviera cheeses (Table 2). Selection of representative isolates of typical *E. durans* was random but attentive to represent all biotypes (2A-2D), while particularly the isolate KE108 (2B) was selected because it demonstrated remarkable antilisterial activity in the agar overlay test only. Conversely, selection of typical *E. faecium* representative isolates within the biotypes 1A-1D was primarily based on their ability to cause strong *in vitro* enterocin (Ent+) inhibition of *L. monocytogenes* (the six bold-written isolates in Table 5). Totally nine typical *E. faecium* isolates from the NSC-cheeses and three from the CSC-cheeses plus the atypical strain *E. faecium* KE77 isolated from the NSC-cheese batch A, were strong Ent + producers. Amongst them was the multiple-Ent + *E. faecium* KE82 (Table 5) recently shown to possess the structural enterocin A, B and P genes (Vandera et al., 2017). Therefore, KE82 served as an Ent + *E. faecium* control that was *a priori* considered a different 'strain' from the non-antagonistic KE85, KE112 and all the other *E. faecium* isolates in biotype 1D (Table 5). Indeed, the SDS-PAGE analysis of whole-cell protein profiles confirmed a distinct strain status for KE82 and the other Ent + representatives except for the KE64 and KE67 isolates (biotype 1B) which shared an identical protein profile (Fig. S1).

Table 5
Biochemical differentiation of 200 LAB isolates in biotypes, numerical distribution of the isolates of each strain-biotype in the Graviera cheeses produced by natural starter culture (NSC) or commercial starter culture (CSC) and summary of the coded/selected 45 representative isolates.

Species identified	Strain biotype	Differentiating sugar biotype profiling								Number of isolates per strain biotype			Representative isolates per biotype subjected to SDS-PAGE and/or molecular identification[a]
		Ara	Man	Sor	Suc	Mel	Tre	Raf	Xyl	NSC	CSC	Total	
E. faecium (Typical)	1A	+	+	−	−	−	+	−	−	2	4	6	**KE118** (Fig. 2; Fig. S1)
	1B	+	+	−	−	+	+	−	−	11	20	31	**KE64, KE67** (Fig. 2; Fig. S1, S2)
	1C	+	+	−	+	−	+	−	−	4	0	4	**K295** (Fig. 2)
	1D	+	+	−	+	+	+	−	−	18	2	20	**KE82,** KE85, KE112 (Fig. 2; Fig. S1, S2)
E. faecium (Atypical)	1E	+	+	+	+	+	+	−	−	2	0	2	KE86 (Fig. 2; Fig. S1)
	1F	+	+	+	+	+	+	+	−	1	0	1	KE102 (Fig. 2; Fig. S1, S2)
	1G	+	−	−	+	+	−	−	−	2	0	2	**KE77** (Fig. 2; Fig. S1, S2)
E. durans (Typical)	2A	−	−	−	−	−	−	−	−	10	2	12	KE96, KE100 (Fig. 2; Fig. S1, S2)
	2B	−	−	−	−	−	+	−	−	3	0	3	KE110, KE108 (Fig. 2; Fig. S1, S2)
	2C	−	−	−	−	+	−	−	−	11	0	11	KE69, KE106 (Fig. 2; Fig. S1)
	2D	−	−	−	−	+	+	−	−	6	3	9	KE66, KE91 (Fig. 2; Fig. S1)
E. durans (Aypical)	2E	−	+	−	−	−	−	−	−	3	0	3	KE98 (Fig. 2; Fig. S1)
	2F	−	−	+	−	−	−	−	−	1	0	1	KE80 (Fig. 2; Fig. S1)
	2G	−	−	+	−	−	+	−	−	1	0	1	KE116 (Fig. 2; Fig. S1)
	2H	−	−	+	−	+	+	−	−	1	0	1	KE88 (Fig. 2; Fig. S1, S2)
	2I	−	−	−	−	+	+	−	+	1	0	1	KE78 (Fig. 2; Fig. S1)
	2J	−	−	−	+	−	−	−	−	1	0	1	KE79 (Fig. 2; Fig. S1)
	2K	−	−	−	+	−	+	−	−	1	0	1	KE94 (Fig. 2; Fig. S1, S2)
E. faecalis (Typical)	3A	−	+	+	+	+	+	−	−	3	0	3	K293, K313, K315 (Fig. 2)
Lb. paracasei	Biotype-I	Homogeneous sugar profile of B-I given in Table 2								2	42	44	H1, H7, H13, H17, H22, H26 (Fig. 3; Fig. S4, S6)
	Biotype-II	Homogeneous sugar profile of B-II given in Table 2								2	4	6	H11, H14 (Fig. 3; Fig. S4, S6)
Lb. plantarum		Homogeneous sugar profile-single biotype in Table 2								5	17	22	H4, H21, H24, H25, H30 (Fig. 3; Fig. S4, S5)
Lc. lactis subsp. *lactis*	W1/Arg+	−	−	−	−	−	+	−	+	1	0	1	KE105 (Fig. 2; Fig. S1, S3)
	W2/Arg+	−	+	−	+	−	+	−	+	1	0	1	KE109 (Fig. 2; Fig. S1, S3)
	W3/Arg-	−	−	−	−	−	+	−	−	2	0	2	Not genotyped at the subspecies level
	CS/Arg+	−	−	−	−	−	+	−	−	0	3	3	Not genotyped at the subspecies level
S. thermophilus	CS-strain	Typical sugar profile of the species given in Table 2								0	3	3	H6, H8 (Fig. S4)
Leuc. mesenteroides	5 biotypes	Heterogeneous-intermixed profiles given in Table 2								5	0	5	Not genotyped at the species/ subspecies level
Total biotypes/ isolates	32									100	100	200	Total representative isolates: 45

[a] The figures or supplementary figures with additional identification data for one or more representative isolates of each biotype are given in parenthesis in the last column. Strong enterocin-producing isolates are indicated in bold. Abbreviations: W, wild strain; CS, commercial starter strain; Arg+, arginine-positive.

Overall, cellular protein profiling confirmed a distinct strain status for all atypical *E. faecium* biotypes and mainly the six atypical *E. durans* biotypes 2F–2K which included single isolates. However, some representative isolates amongst the typical *E. faecium* and *E. durans* biotypes displayed intermixed protein profiles at the strain level (Fig. S1).

Two indigenous strains of *Lc. lactis*, KE105 and KE109, isolated from NSC-cheeses were also selected because although they possessed a typical (arg+, xyl+) phenotype of the subspecies *lactis* (Table 5), they could be wild *cremoris* strains, possibly relating with our novel, nisin A-producing M78 and M104 strains from Greek raw milk (Parapouli et al., 2013). Therefore, KE105 and KE109 isolates were first tested by the SDS-PAGE method which confirmed that they were different *Lc. Lactis* strains with distinct protein profiles (Fig. S1). Finally, selection of representative isolates of *Lb. plantarum* (5) and *Lb. paracasei* biotype I (6) and biotype II (2) also was random because all three biotypes were homogeneous (Table 2) and none included bacteriocinogenic isolates (Table 5). In summary, only 7 out of 32 strain biotypes (10 isolates; 5% of total) were not subjected to molecular id analyses (Table 5); unselected remained mainly the five very heterogeneous and quite intermixed biotypes of *Leuconostoc mesenteroides* (Table 2) because leuconostocs generally were subdominant in the NSC-cheeses and undetectable in the CSC-cheeses (Table 3).

3.5. Polyphasic identification of representative Graviera cheese LAB isolates by genotypic methods

Consistent with their biochemical species identification, the presence of three distinct IGS profiles, clearly separating the representative isolates of all typical and atypical strain biotypes of *E. faecium* (Fig. 2A), *E. durans* (Fig. 2B) and *E. faecalis* (Fig. 2D) were obtained. Exception was strain KE102 which, in accordance with its distinct biotype (Table 5), displayed an IGS profile distinct from all other *E. faecium* isolates which had a higher similarity with the IGS profile of the *E. faecalis* biotype 3A isolates (Fig. 2D). Nevertheless, IGS patterns *per se* can neither ensure the LAB species identification nor distinguish on the strain level. For the above purposes, eight finally selected representative strains of the diverse *E. faecium* and *E. durans* biotypes plus the 'unidentified' strain KE102 by IGS (Table 5; Fig. 2) were submitted for 16S rRNA gene sequencing analyses. All representative isolates of *E. faecium* and *E. durans* clustered with reference strains of *E. faecium* and *E. durans*, respectively, while the strange strain KE102 clustered with *E. faecium* rather than with *E. faecalis*, *E. hirae* or other *Enterococcus* spp. in the phylogenetic tree (Fig. S2).

As it was expected, the two wild *Lc. lactis* strains KE105 and KE109 shared an IGS profile that was distinct from all *Enterococcus* representatives tested comparatively on the same gel (Fig. 2C). Both strains were genotyped as *Lc. lactis* subsp. *lactis* by the subspecies-specific PCR method (Fig. S3; Table S1).

As also it was expected, all five representative isolates of the single *Lb. plantarum* biotype (Table 5) shared an IGS pattern distinct from those of the representative isolates of *Lb. paracasei* biotypes I and II (Fig. S4) while the IGS method further distinguished both *Lactobacillus* species from the two representative isolates of the CS-strain of *S. thermophilus* (Table 5) tested on the same gel for comparison (Fig. S4). In order

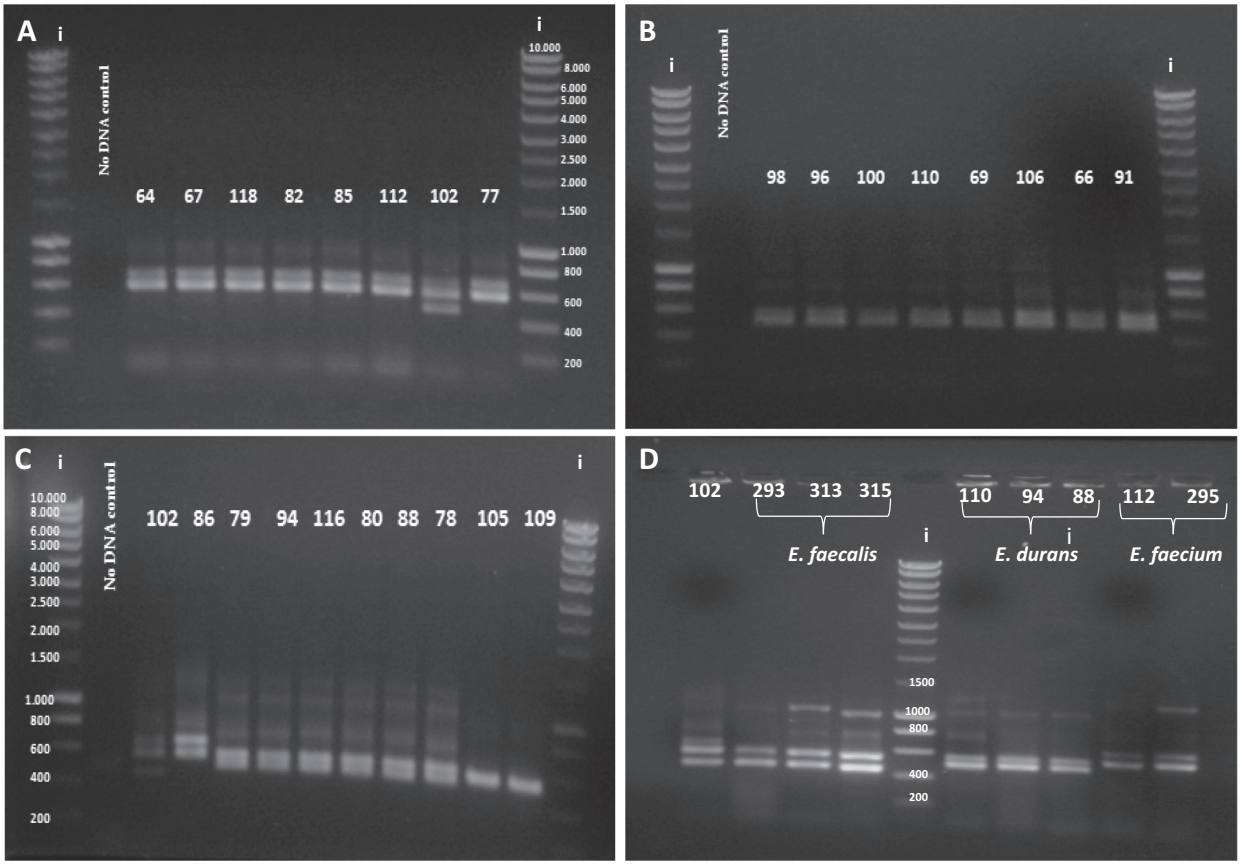

Fig. 2. Intergenic spacer (IGS) profiles of 27 representative isolates of *Enterococcus* spp. from Graviera cheese. A: Typical (64, 67, 118, 82, 85, 112) and atypical (102, 77) *E. faecium* isolates; B: Atypical (98) and typical (96, 100, 110, 69, 106, 66, 91) *E. durans* isolates; C: Atypical (102, 86) *E. faecium* isolates compared with atypical (79, 94, 116, 80, 88, 78) *E. durans* isolates and the two wild *Lactococcus lactis* subsp. *lactis* strains (105, 109); D: Comparison of the species-specific IGS profile of typical *E. faecalis* (293, 313, 315) isolates with that of typical *E. faecium* (112, 295) and typical (110) and atypical (94, 88) *E. durans* isolates, plus the most atypical single strain 102. i: SmartLadder (Eurogentec). Discrimination of the 27 isolates representing a total of 113 *Enterococcus* isolates (Table 2) in 19 'strain biotypes' are presented in Table 5. The isolate code prefixes KE/K were omitted on the figure photos for simplification.

for the NSLAB lactobacilli to be distinguished at the strain level, a multiplex-PCR (Table S1) was first used to ensure identification of the five representative isolates of *Lb. plantarum* (Fig. S5), and a PCR reaction using species-specific primers (Table S1) was applied to ensure identification of all representative isolates of biotypes I and II in Table 5 as *Lb. paracasei* (Fig. S6). Afterwards intra-species variations were detected by generating the RAPD profiles using as template gDNA of all 13 representative *Lactobacillus* isolates (Fig. 3). All five *Lb. plantarum* representatives gave the same RAPD profile (Fig. 3), in agreement with their species phenotypic homogeneity (Table 2). Thus, probably one *Lb. plantarum* strain prevailed in the Graviera cheeses of this study. The RAPD method (Fig. 3) also differentiated the representative isolates H11 and H14 of the minor atypical (sorbitol-negative) *Lb. paracasei* biotype II from those of the major typical (sorbitol-positive) *Lb. paracasei* biotype I (Table 5). In specific, the H11 and H14 isolates shared the same RAPD profile, which suggested that they might be duplicate isolates of one *Lb. paracasei* biotype II strain (Fig. 3). In contrast, the six representative isolates of *Lb. paracasei* biotype I displayed three distinct RAPD profiles (Fig. 3) despite the fact that all 44 isolates of biotype I were homogeneous biochemically (Table 2). This finding revealed a major intra-species genotypic diversity of *Lb. paracasei* prevailing in mature (day-90) Graviera cheeses; also it showed that LAB isolates that seem to be identical phenotypically may cluster in different groups by RAPD or other molecular discriminative tools at the strain level.

4. Discussion

This study compared the microbial stability, safety and LAB ecology of mature Greek Graviera cheese manufactured from commercially thermized ewes'/goats' milk mixtures supplemented with either NSC or CSC. Fresh, yogurt-like NSCs had an approximate 2.5-log lower initial mixed-LAB cell density than the freeze-dried CSC, which might be an important technological reason for the absence of *S. thermophilus* and *Lb. bulgaricus* isolates from the NSC-cheeses after 90 days of ripening. Nevertheless, both RTE Graviera cheese types were microbiologically safe, irrespective the type of starter used for milk acidification and potential variations in the prevalence rates of the SLAB during cheese fermentation; pathogenic bacteria were absent or below the maximum permitted levels specified in EC Regulation (European Commission, 2007) for ripened cheeses made from heat-treated milk. Similar results, particularly regarding the microbial safety of Graviera cheeses produced with CSCs, were reported in previous studies and included validation of artificially contaminated CSC-cheeses with *L. monocytogenes* and enterotoxigenic *S. aureus* (Giannou et al., 2009a; Samelis et al., 2009b, 2014). The NSC-cheeses of this study were also microbiologically safe, evidently because raw milk was thermized before cheese manufacture (Samelis et al., 2009a). The only safety concern was the high prevalence in all NSC-ripened cheese batches of indigenous strains of the *E. faecium/durans* genomic group (Tables 1–5). Evidently most enterococci were raw milk contaminants that had survived thermization and possibly predominated in the fresh NSC-cheese curds during fermentation under the low competitiveness of the non-concentrated, acid-stressed yogurt-like NSCs (Samelis et al., 2009a; Vandera et al., 2018). Whether these indigenous enterococci represent a true cheese safety risk is critically discussed in later paragraphs.

While the identification of 200 isolates indicated the presence in RTE Graviera cheeses of eight of the commonest dairy LAB species (Table 2), the results further revealed the existence of major compositional differences between the NSLAB microbiota prevailing in each cheese type at full maturation. To ensure the identification accuracy of the LAB isolates at the species-subspecies level, a culture-dependent polyphasic approach combining several molecular techniques and the use of species-specific PCR primers was applied. Due to certain technical and fund limitations, all isolates were first discriminated into a minimum number of distinct 'strain biotypes' within each LAB species identified biochemically and then representative isolates of each biotype were identified to confirm their species identification molecularly. Particularly for the 28 representative isolates of 18 different *E. faecium/durans* biotypes plus *E. faecalis* biotype 3A (Table 5), IGS polymorphisms proved a useful molecular tool for their interspecies differentiation, in agreement with previous reports for *Enterococcus* and additional LAB genera by others (Alves et al., 2004; Belgacem et al., 2009; Blaiotta et al., 2002; Morandi et al., 2012). However, neither the IGS profiles nor the phylogenetic identifications of 25 out of 32 LAB biotypes obtained by 16S rRNA gene sequencing or by species-specific PCR methods (Fig. S2–S6) could discriminate the LAB isolates at the genotypic 'strain' level. On the other hand, particularly for the highly heterogeneous *E. faecium* and *E. durans*, potential increases in the number of 'strain genotypes' further to the number of 'strain biotypes' in Table 5 would be meaningless unless such discrimination has importance for cheese technology. As an example, sharing their basic sugar fermentation reactions, the reference m-Ent + *E. faecium* KE82 and its enterocin-negative control KE85 were assignable to biotype 1D isolates (Table 5). However, they proved very different strains not only because strain KE82 is a strong enterocin producer (Section 2.5) but also because it promotes good growth on Baird-Parker agar due to its natural resistance

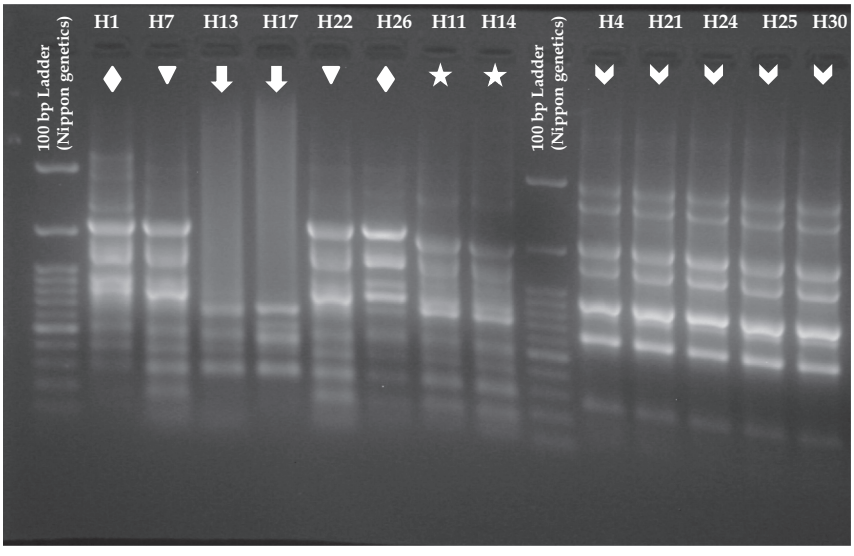

Fig. 3. RAPD profiles of the selected representative isolates of mesophilic *Lactobacillus* spp. determined using primer M13. All five *Lb. plantarum* representative isolates (H4, H21, H24, H25, H30) shared an identical RAPD profile, as also did the two representative isolates (H11, H14; indicated with asterisk) of the minor (sorbitol-negative) biotype II of *Lb. paracasei* in Table 5. In contrast, the six representative isolates of the major (sorbitol-positive) homogeneous biotype I of *Lb. paracasei* (Table 5) formed three distinct RAPD profiles indicated by the three different symbols (arrow, triangle, rhomb), respectively.

to tellurite, produces hydrogen peroxide as shown by its α-hemolysis reaction on 5% sheep blood agar and ferments gluconate (Vandera et al., 2018); additionally, its cellular protein profile did not match that of strain KE85 (Fig. S1), while the two strains clustered quite distantly within the *E. faecium* group in the 16S rRNA phylogenetic tree (Fig. S2). In conclusion, the total number of 32 'strain biotypes' in Table 5 may increase upon genotyping of more LAB isolates at the strain level, but it cannot decrease in a manner to alter the main ecological and practical technological findings of this study.

The predominance in all CSC-ripened cheeses of *Lb. paracasei* subsp. *paracasei* and *Lb. plantarum* strains was consistent with our previous Graviera cheese ecology studies (Samelis et al., 2010, 2011) and in general agreement with numerous studies and reviews by others on the NSLAB microbiota of traditionally ripened European hard cheeses (Beresford et al., 2001; De Angelis et al., 2001; Dolci et al., 2008; Gobbetti et al., 2015; Montel et al., 2014; Settanni and Moschetti, 2010). Particularly for the CSC-Graviera cheeses, once *Lb. paracasei* (biotypes I and II) along with *Lb. plantarum* or potentially other closely related mesophilic *Lactobacillus* spp. are established for growth after 4 to 5 weeks of ripening at temperatures 16–19 °C (Noutsopoulos et al., 2017; Samelis et al., 2010, 2011), they would predominate in mature RTE Graviera cheeses after 12 weeks of ripening (Tables 2 and 3). Litopoulou-Tzanetaki and Tzanetakis (2011) also reported predominance of *Lb. paracasei* and *Lb. plantarum* in mature PDO Graviera Kritis raw milk cheeses. However, few or no isolations of these mesophilic NSLAB lactobacilli were recovered from fresh (day-1) Graviera Kritis cheeses, from which mainly wild lactococci, enterococci (*E. gallinarum, E. faecium and E. faecalis*) and leuconostocs (*Leuc. lactis*, *Leuc. mesenteroides*) were isolated (Bozoudi et al., 2016). The above authors concluded that leuconostocs may attribute greatly to the opening of the small holes in the Graviera Kritis cheese core by their gas-forming biochemical activities during fermentation, while like us emphasized that some of their isolates of the *Leuc. mesenteroides* complex might have been misidentified due to their very high similarity of the 16S rRNA gene sequence (Bozoudi et al., 2016). Technologically, however, a major difference in the NSLAB ecology between naturally-fermented Graviera cheese varieties produced from raw milk *vs.* thermized milk becomes evident: while the fresh (day-1) PDO Graviera Kritis cheeses were far more enriched in wild lactococci and leuconostocs due to the use of raw ewes' cheese milk (Bozoudi et al., 2016), thermization (60 °C, 30 s) of the raw ewes'/goats' milk mixtures before Graviera cheese processing in Epirus, Greece, reduced the isolation frequency of wild lactococci and leuconostocs from 20.4% and 18.4% in raw milk to 4% and undetectable levels in the corresponding thermized cheese milk, respectively, whereas the prevalence of indigenous thermotolerant *Enterococcus* isolates increased from 40.8% in raw milk to 72% in thermized milk (Samelis et al., 2009a). Then, from the fresh Graviera Kritis raw milk curd (day-1) fermentation to full cheese maturation (day-90), lactococci, leuconostocs and enterococci declined in favor of *Lb. paracasei* and *Lb. plantarum* (Litopoulou-Tzanetaki and Tzanetakis, 2011), which in the case of Graviera cheeses made from thermized milk predominated in the CSC-cheese batches only (Table 2). Additional studies are required to elucidate why and how the prevalence of NSLAB mesophilic lactobacilli was prevented in favor of indigenous *E. faecium/durans* and generally realize the different type of SLAB and NSLAB interactions during processing of NSC-fermented Graviera cheeses.

The results of this study further indicated that compared with the atypical sorbitol-negative single-strain biotype II of *Lb. paracasei*, at least three different strains of the typical sorbitol-positive biotype I had a selective growth advantage in the core of CSC-fermented Graviera cheeses during ripening. Regarding the intra-species diversity of *Lb. paracasei* herein revealed by RAPD profiling (Fig. 3), similar observations were reported for 27 *Lb. paracasei* subsp. *paracasei* isolates from four traditional Greek cheeses, which formed three distinct clusters with an internal correlation coefficient ranging from 55.4 to 62.6% (Mama et al., 2002). Intra-species heterogeneity was reported for *Lb. paracasei* and other closely related nonstarter isolates of the *Lb. casei/paracasei* group from Italian ewe cheeses (De Angelis et al., 2001). Whether this high heterogeneity of *Lb. paracasei* would be decisive for genotype (strain)-specific *in situ* competitiveness and biochemical activities in different cooked hard cheese products or processing plants requires further investigations. Due to their superior growth dynamics, complex interactions with SLAB and technological importance in traditional hard or semi-hard cheese fermentations (Desfossés-Foucault et al., 2013; Gobbetti et al., 2015; Levante et al., 2017), members of the *Lb. casei/paracasei/rhamnosus* genomic group are currently marketed as SLAB and also are amongst the most promising probiotics for milk-based products (Settanni and Moschetti, 2010). Also there is a recent, highly increasing interest on the commercial use as SLAB of selected indigenous *Lb. plantarum* strains with probiotic potential to enhance quality and safety of traditional PDO Greek cheeses, such as Feta cheese (Papadopoulou et al., 2018). Additional molecular studies are therefore required to determine potential differences in the origin, residence, prevalence and spatial distribution of the present or other genotypes of the *Lb. casei/paracasei* and *Lb. plantarum* genomic groups in Graviera cheese products and the processing plant environment.

The most important finding of this study was the predominance of members of the *E. faecium* group, as opposed to a very low occurrence of *E. faecalis*, in mature RTE Graviera cheeses fermented with yogurt-like NSCs (Tables 1–5). The present results extended our preliminary findings (Giannou et al., 2009b) on this critical issue because of the controversial status of dairy enterococci which continues to exist up to date (Gaglio et al., 2016; Popović et al., 2018). High levels of indigenous enterococci are still perceived with skepticism as regards cheese safety, owing to their opportunistically pathogenic nature and potential transfer to foods of hemolytic, virulent and multidrug resistant strains of clinical origin (Hammad et al., 2015; Johnson et al., 2010; Moreno et al., 2006). Therefore, application of enterococci as commercial starter, costarter or adjunct cultures in the dairy industry is still debated and their legal status is pending, although three *Enterococcus* species have been included in the list of beneficial microorganisms in food fermentations (Bourdichon et al., 2012): *E. faecium*, its close genomic relative *E. durans* and less frequently *E. faecalis* have been acknowledged for their natural occurrence, occasional dominance and functionality in various types of Southern European traditional raw milk cheeses since the 1990s' (Domingos-Lopes et al., 2017; Fuka et al., 2017; Gaglio et al., 2016; Giannou et al., 2009b; Giraffa, 2003; Litopoulou-Tzanetaki and Tzanetakis, 2011; Moreno et al., 2006; Settanni and Moschetti, 2010; Terzić-Vidojević et al., 2015). Artisanal raw or thermized milk cheeses are still consumed in local or urban European markets without cause of safety problems or implications. Furthermore, the most recent of the aforementioned studies based on molecular methods, have validated that generally harmless strains of *E. faecium*, *E. durans*, *E. faecalis* plus *E. lactis* and *E. italicus*, two recently described dairy species (Fortina et al., 2008; Morandi et al., 2012), are involved in traditional cheese fermentations. Such strains most likely are specific of milk farms or may become endemic in small dairies of rural areas still applying traditional manufacturing practices in artisanal cheeses, which are safe at least as regards autochthonous enterococci. Supportively, in reviewing safety assessment of the dairy *Enterococcus*, Ogier and Serror (2008) concluded: 'no correlation has yet been demonstrated between ingestion of food products containing enterococci and infection'. To the best of our knowledge, this vital safety conclusion remains valid; no documented cheese (food) poisoning cases, not even alarming RT-PCR studies on risky virulence gene expressions *in situ* in cheese particularly implicating *E. faecium* or *E. durans* (the dominant species in the NSC-ripened Graviera cheeses), occur. Therefore, the *in situ* expression of virulence genes, commonly detected in traditional cheese isolates of *E. faecium*, *E. durans* or other *Enterococcus* co-habitants (Domingos-Lopes et al., 2017; Fuka et al., 2017; Gaglio et al., 2016; Hammad et al., 2015; Terzić-Vidojević et al., 2015), is likely to occur only when the surrounding environment is

stressful enough to trigger underlying virulence mechanisms and potentially associated, cross-protective stress responses in dairy enterococci (Popović et al., 2018; Samelis and Sofos, 2003). In this aspect, several undesirable enterococcal virulence traits are probably of enhanced activation upon nosocomial infections (Johnson et al., 2010) or after intense antibiotic treatments of infected animals in dairy farms. Otherwise the gene clusters associated with opportunistic enterococcal pathogenesis remain 'silent' or encode non-virulent cellular functions, such as biofilm formation ability, when the environmental stresses are mild, as it happens in synthetic culture media *in vitro* (Popović et al., 2018). In specific, natural Graviera or similar traditional cheese environments of mild acidity (pH > 5.0) and of generally low imposed (acid) stresses on the indigenous microbiota are very unlikely to trigger the expression of dormant virulence genes in dairy *Enterococcus* strains and support the evolution of harmful pathogenic mutants, provided raw or thermized cheese milks originate from naturally-fed ruminants and are free of drugs or other chemical preservatives. Furthermore, Gaglio et al. (2016) reported that *E. faecium*, *E. durans* and *E. casseliflavus* without any type of risk determinants were isolated from traditional Italian PDO stretched cheeses. Thus, similar completely harmless *Enterococcus* genotypes may be present amongst our Graviera cheese isolates.

5. Conclusions

Members of the *E. faecium* group predominated in mature Greek Graviera cheeses manufactured in a small traditional dairy plant of Epirus from thermized ewes'/goats' milks with craft-made natural yogurt-like starters. Because thermization of raw milk selects for lactose-fermenting *Enterococcus* spp., survivors have a profound ability for growth during thermized cheese milk curdling, curd cooking and fermentation at temperatures 34–48 °C unless the SLAB outcompete them. Otherwise, dairy enterococci, mainly *E. faecium* and *E. durans*, have great chances to predominate even in the ripened RTE products, as it was shown for the NSC-cheeses of this study. More recent PCR-based studies confirmed selective isolations of m-Ent + *E. faecium* biotypes, with an *ent*A-*ent*B-*ent*P or *ent*A-*ent*B profile, from bulk commercially thermized milk after a culture enrichment in simulation of the natural Graviera cheese fermentation process (37 °C, 48 h); no β-hemolytic, cytolysin-positive or other-type of Ent + *E. faecalis* were detected in any of the above culture-enriched milks simultaneously (Vandera et al., 2018). The high natural selection and growth prevalence of *E. faecium* in the NSC-ripened Graviera cheeses, including several Ent + strains, may indicate its higher resistance to milk thermization and better adaptation than *E. faecalis* during fermentation and ripening. In contrast, the predominance of *E. faecium* and other indigenous *Enterococcus* spp. in the CSC-ripened cheeses was prevented, evidently because of the high competitive growth effects of concentrated CSCs, especially *S. thermophilus* (Noutsopoulos et al., 2017). While technological and safety concerns associated with the poor performance of non-concentrated NSCs suggest that concentrated CSCs should be applied in traditional Greek Graviera cheese productions, sensory panel evaluations indicated that the NSC-cheeses, which were ripened almost exclusively by dairy members of the *E. faecium* genomic group, were of worse appearance but sometimes of better flavor than the CSC-ripened cheeses. Therefore, the microbial (LAB) community of traditional NSC-Graviera cheeses from thermized milk needs to be investigated further by culture-independent approaches during processing and in correlation with physicochemical and sensorial attributes analyzed by objective methods for cheese color, texture and aroma determination and product discrimination, as recently studied for the PDO Greek Graviera Kritis and Naxou cheeses (Bozoudi et al., 2016) and, for instance, Swiss-type Maasdam cheese (Duru et al., 2018). Additional studies are in progress to characterize numerous beneficial autochthonous SLAB/NSLAB isolates collected all across the natural fermentation and ripening of NSC-Graviera cheeses. Our research is focused to characterize technologically harmless dairy *Enterococcus* spp. plus wild *Lc. lactis*, *Lb. plantarum*, *Lb. paracasei* and *Leuconostoc* strain genotypes and conduct strain-by-strain validation studies of beneficial Ent + and m-Ent + *E. faecium* strains for application as novel, craft-made, costarter or protective adjunct cultures in traditional Greek cheeses.

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