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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