Assuring Growth Inhibition of Listerial Contamination during Processing and Storage of Traditional Greek Graviera Cheese: Compliance with the New European Union Regulatory Criteria for Listeria monocytogenes

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ABSTRACT

The current microbiological regulatory criteria in the European Union specify a maximum Listeria monocytogenes population of 100 CFU/g allowable in ready-to-eat foods provided the product will not exceed this limit throughout its shelf life. The aim of this study was to validate the manufacturing method for traditional Greek Graviera cheese produced from thermized milk. Initial challenge experiments evaluated the fate of inoculated L. monocytogenes (ca. 4 log CFU/ml, three-strain cocktail) in thermized Graviera cheese milk (TGCM; 63°C for 30 s) in the presence and absence of a product-specific starter culture (SC) in vitro. Milk samples were incubated for 6 h at 37°C and then for 66 h at 18°C. Experiments were conducted to evaluate the fate of a cocktail of three nonpathogenic L. monocytogenes and L. innocua indicator strains inoculated (ca. 3 log CFU/g) in Graviera cheeses commercially manufactured from TGCM+SC. Cheeses were brined, ripened at 18°C and 90% relative humidity for 20 days, and stored at 4°C for up to day 60 under vacuum. In TGCM, L. monocytogenes increased by ca. 2 log units, whereas in TGCM+SC L. monocytogenes growth was retarded (P < 0.05) after a ca. 1-log increase within 6 h at 37°C. Populations of Listeria indicator strains did not grow in TGCM+SC cheeses at any stage; they declined 10-fold in fresh cheeses within 5 days and then survived with little death thereafter. Thus, growth inhibition but not inactivation of potent natural Listeria contaminants at levels below 100 CFU/g occurs in the core of traditional Greek Graviera cheese during fermentation, ripening, and storage.

The new microbiological criteria in the European Union (EU) Regulation 2073/2005 (10) and in its 1441/2007 modification (11) specify a maximum Listeria monocytogenes population of 100 CFU/g (or ml) allowable in food products both when they are ready to be placed on the market and during their shelf life. This criterion has replaced the zero tolerance requirement in ready-to-eat (RTE) foods unable to support growth of L. monocytogenes (i.e., foods with a pH of ≤4.4 and a water activity [aw] of =0.92, or a pH of ≤5.0 and an aw of ≤0.94, or a shelf life of ≤5 days). However, this criterion applies to any RTE food for which the manufacturer is able to demonstrate to the satisfaction of the competent authority that the product will not exceed the limit of 100 CFU/g throughout its shelf life (10, 11). Such validation studies are not presently available for most traditional Greek cheeses produced from raw or thermized milk in small dairy plants. These cheeses may contain a few L. monocytogenes cells, thus failing to comply with the alternative criterion “absence in 25 g” when ready for sale. L. monocytogenes has been detected in various retail cheese products (18, 21, 23, 24, 31), including traditional Greek cheeses (12, 34), and has caused several fatal outbreaks mostly linked to soft cheeses (1, 9, 32). Listerial contamination occurs either as a result of the use of contaminated raw milk (4, 25, 37) or as a result of cross-contamination during and after cheese processing (1, 14, 27, 30–32). Thus, to assure that L. monocytogenes contamination will not exceed the 100 CFU/g allowance in the final product, it is necessary to evaluate the behavior of this pathogen in the cheese core during processing and on the cheese surface postprocessing.

Graviera is the finest and most popular Greek hard cheese produced mainly from ewe’s milk, which is either pure or mixed with 10 to 30% goat’s milk (15, 22). Fresh cheeses of average size (30 to 35 cm in diameter and 12 to 15 kg in weight) are ripened for 3 months and then portioned and packaged for retail distribution and storage (2). Mini Graviera cheeses (10 to 12 cm in diameter and ca. 1 kg in weight) ripened for 3 to 4 weeks are produced commercially because they are convenient RTE products easily stored in homes. When ready for sale, Graviera cheeses should have a maximum water content of 38 to 40% and a minimum fat content in dry matter of 40% (2). Their pH and aw values range from 5.2 to 5.6 and 0.94 to 0.96, respectively (15, 19); thus, according to the EU specifications for RTE foods, Graviera cheeses may theoretically support growth of L. monocytogenes. However, in a recent study (15) we found that L. monocytogenes was unable to grow on the surface of fully ripened Graviera cheese when introduced postprocessing, regardless of packaging (air or vacuum) and storage temperature (4, 10, or 25°C). Thus,

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matured Graviera cheeses that may be accidentally contaminated at retail with the EU maximum allowed *L. monocytogenes* level of 100 CFU/cm² are at low risk for supporting growth of this pathogen (15). However, the growth potential of *L. monocytogenes* in fresh and ripening Graviera cheeses to levels that might exceed 100 CFU/g in the retail product has yet to be ascertained, given the high survival potential of the pathogen on the RTE cheese product, especially at 4°C under vacuum (15).

The present study was undertaken to evaluate the fate of *Listeria* in the core of Graviera cheese during processing and storage. Because use of pathogenic *L. monocytogenes* strains in the commercial plant was not permitted, in vitro challenge thermized milk experiments were first conducted in the laboratory with pathogenic strains, and then nonpathogenic *Listeria* indicator strains were used for artificial contamination of freshly prepared commercial cheeses.

**MATERIALS AND METHODS**

**Bacterial strains.** Six strains of *Listeria* were used. A three-strain composite of pathogenic *L. monocytogenes* strains, Scott A (clinical isolate, serotype 4b), ISS G79 (soft cheese isolate, serotype 1/2b), and ISS G185 (blue-veined cheese isolate, serotype 1/2a) (Dr. Paolo Aureli, Instituto Superiore di Sanita, Rome, Italy) (15, 16), was used in all thermized milk challenge experiments conducted in vitro in our laboratory. Another three-strain composite of *Listeria innocua* LMG 11387, *L. innocua* M58 isolated from raw milk (36), and a nonpathogenic *L. monocytogenes* strain (no. 10, serotype 4ab, Prof. J. Farkas, Szent Istvan University, Budapest, Hungary) was used in all commercial Graviera cheese inoculation trials conducted in a local dairy plant (Pappas Bros., Filippia, Epirus, Greece). Preliminary experiments confirmed that all three nonpathogenic strains were similar enough to pathogenic *L. monocytogenes* to serve as indicators of the pathogen’s behavior in cheese. Strain 10 was originally described by Ralovich (28) as avirulent in Anton’s and mouse tests and was previously used to artificially contaminate sausage meat trimmings under commercial in-plant conditions (35). In addition, both strains 10 and M58, which are not from bacterial type culture collections, were confirmed by the API *Listeria* identification kit (bioMérieux, Marcy l’Etoile, France) as sharing the API identification code 7510 (36), which identifies *L. innocua* and corresponds to the commonest “twin” API code, 6510, for *L. monocytogenes*, which includes strain Scott A (data not shown).

All strains were available as frozen stock cultures (−30°C) in tryptic soy broth containing 0.6% yeast extract (LAB M, Bury, Lancashire, UK) and 20% glycerol and were activated and subcultured twice at 30°C for 18 to 22 h in 10 ml of brain heart infusion (BHI) broth (LAB M) before use in the experiments.

**Preparation of *Listeria innocua* inocula.** Each of the strains was cultured in 10 ml of BHI broth at 30°C for 20 to 22 h, the cells were harvested by centrifugation (3,200 × g for 15 min), and the pellet was resuspended in 10 ml of sterile Ringer solution (Merck, Darmstadt, Germany). Suspended cells of the pathogenic *L. monocytogenes* strains were combined, and the same protocol was used for the nonpathogenic *L. monocytogenes* and *L. innocua* strains. For each type of challenge experiment, the mixed cell suspension of the respective three-strain composite was serially diluted with Ringer solution to the desired inoculation level either in thermized milk or in fresh Graviera cheese curds. To confirm the population level, the inoculum was plated on tryptic soy agar (LAB M) containing 0.6% yeast extract and PALCAM agar (Merck) or on Agar *Listeria* Ottaviani Agosti (ALOA; AES120 Laboratoire, Bruz, France) and incubated for 48 h at 30°C.

**Thermized milk challenge experiments.** Thermized milk samples (1 liter) were obtained from the participating cheese plant, which specializes in traditional Graviera cheese production. Samples were taken from the composite bulk milk (ca. 3 ton, 90% ewe’s milk and 10% goat’s milk) after thermization at 63°C for 30 s but before the beginning of Graviera cheese processing. The milk samples were transported to the laboratory in insulated iceboxes and analyzed microbiologically and for pH within 30 min after transportation. The thermized milk samples were then distributed in 100-ml aliquots in presterilized 250-ml Duran flasks and inoculated with ca. 4 log CFU/ml of the composite of pathogenic *L. monocytogenes* strains. Half of the samples were further inoculated with 2 mg/100 ml of a commercial freeze-dried starter culture (SC) consisting of a mixture of lactic acid bacteria (LAB) strains of *Streptococcus thermophilus*, *Lactococcus lactis* subsp. *lactis*, and *L. lactis* subsp. *lactis* var. *diacetylactis* and *lactococci* (GR02, Mofin Alce Group, Novara, Italy). The SC was added to evaluate the behavior of *L. monocytogenes* in thermized Graviera cheese milk (TGCM) without or with the SC, which is routinely used commercially. The level of inoculation of TGCM samples with the SC was proportional to the recommended level in the bulk milk to yield an initial mixed LAB starter population of ca. 6 log CFU/ml. Inoculated samples were incubated at 37°C for 6 h and then at 18°C for another 66 h to simulate the conditions for milk curdling and cheese ripening, respectively. Samples (5 ml) were taken from each flask at 0, 3, 6, 12, 24, 48, and 72 h for microbiological analyses and pH determination.

**Graviera cheese challenge experiments.** Mini Graviera cheeses (ca. 1 kg each) were manufactured in the cheese plant. Taking into account the fact that artificial contamination, even with nonpathogenic *Listeria* species, was not allowed across the commercial processing line, a special procedure was followed. Large-scale cheese manufacture was initiated as usual by pouring 1,500 to 2,000 liters of cooled (32 to 34°C) bulk milk after thermization (63°C for 30 s) into a heat-jacketed stainless steel cheese vat. The commercial SC (50 U/1,000 liters of milk) and then the rennet was added (40 g of rennet powder per 1,000 liters of milk at 34°C for 35 min). After curdling, the curd was cut in 2.5-cm cubes and cooked under a standard in-plant protocol to a final temperature of 48°C. The cooking process included gradual heating of the curd from 34 to 48°C within 33 min, followed by constant heating at 48°C for another 15 min with continuous stirring. At this stage, 10 kg of curd (pH 6.3 ± 0.2) in its whey was rapidly transferred to the commercial plant’s pilot area in a stainless steel vessel, and half of the whey was drained out immediately. The curd was inoculated with the three-strain nonpathogenic *Listeria* composite at ca. 3 log CFU/g, stirred vigorously, left for 3 min for inoculum attachment, and distributed into polyvinyl chloride cheese molds (12 cm in diameter), which were perforated on all sides with the top open and slightly wider than the bottom. Curd distribution was enhanced by direct immersion of the clean cylindrical molds into the vessel. The filled molds were lifted to strain off most of the remaining whey, placed the inside another mold, pressed manually to form the mini cheeses, and drained well. The fresh cheeses were kept at 18°C overnight, immersed in 20% brine at 12°C for 24 h, drained at 12°C for another 24 h, and then transferred to a controlled pilot.
Microbiological composition and quality of the thermized milk and the fresh cheese curds were evaluated before inoculation with Listeria. Milk or cheese samples (10 ml or 10 g) were analyzed in duplicates for total mesophilic bacteria, mesophilic and thermophilic LAB, enterococci, catalase-positive cocci, pseudomonads, enterobacteria, yeasts, and coagulase-positive staphylococci by direct plating. The presence of naturally occurring Listeria species and/or L. monocytogenes was evaluated by culture enrichment in 25 ml of milk or 25 g of cheese. The media and methods used were as described by Bontinis et al. (6). Unless otherwise stated, all media and supplements were purchased from LAB M.

For the periodic analyses of Listeria-inoculated TGCM or mini cheese samples, 1 ml of milk or 10 g of cheese was transferred to 9 or 90 ml of 0.1% (wt/vol) buffered peptone water (BPW) in tubes or stomacher bags, respectively. Samples on mini cheeses were collected with presterilized cork borers (1-cm diameter). Each sample was from duplicate cores taken from one cheese, and two mini cheeses were sampled per sampling day. Milk samples were homogenized by vortexing, and cheese samples were homogenized in a stomacher (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 was spread in duplicate on agar plates.

For TGCM samples, L. monocytogenes was enumerated on PALCAM agar incubated at 30 °C for 48 h, and total LAB were enumerated on M-17 agar incubated at 37 °C for 48 h. Indigenous enterococci were expected to be numerous in TGCM because of their natural selection by milk thermization (36); therefore, their populations were selectively enumerated on kanamycin aesculin azide (KAA) agar incubated at 37 °C for 48 h.

For cheese samples, populations of Listeria were enumerated on ALOA incubated at 37 °C for 24 to 48 h. ALOA was used rather than PALCAM agar to ensure that nonpathogenic Listeria colonies without halos survived and to confirm the absence of halo-forming L. monocytogenes in the cheese samples throughout ripening and storage. No opaque halo formation on ALOA due to lack of phospholipase activity was found for L. monocytogenes strain 10, and this finding is consistent with the nonvirulent character and the API-based identification of this strain as L. innocua. For Listeria enumeration, 1 ml from the stomacher bag was plated in quadruplicate on ALOA plates to achieve a lowest detection limit of 1.0 log CFU/g of cheese. Separate enumerations of thermophilic and mesophilic LAB populations in cheese were performed by plating samples on M-17 agar incubated at 42 or 22 °C for 48 or 72 h, respectively, and enterococci were again enumerated on KAA agar incubated at 37 °C for 48 h.

Chemical analyses. The pH of all milk samples during incubation and all cheese samples throughout ripening and storage was measured with a digital pH meter (model 3510, Jenway, Dunmow, Essex, UK) equipped with a glass electrode. The electrode was immersed in milk samples directly and in cheese samples after homogenization with distilled water. The moisture of the cheeses was determined after formulation (day 1), after ripening (day 23), and at the end of storage (day 60) as described previously (6).

Statistical analysis. The behavior of L. monocytogenes in TGCM in the presence or absence of SC was evaluated in two individual experiments, whereas cheese challenge experiments were replicated three times by analyzing two cheese samples per replicate. In all experiments, the microbiological counts were converted to log CFU per milliliter or gram, and the means and standard deviations (SD) were calculated. The data were subjected to a one-way analysis of variance using SPSS for Windows (version 15.0, Microsoft, Redmond, WA), and the means were separated by the least significance difference procedure at the 95% confidence level (P < 0.05).

RESULTS

Behavior of L. monocytogenes in co-culture with the natural and starter LAB flora in TGCM. Before inoculation (day 0), TGCM samples (pH 6.6 ± 0.1) contained 5.2 ± 0.2 log CFU/ml of total LAB flora (Fig. 1), whereas populations of indigenous enterococci were 4.3 ± 0.3 log CFU/ml (Fig. 2). Addition of the commercial SC increased the initial (day 0) LAB populations (P < 0.05) in TGCM+SC samples to 6.2 ± 0.1 log CFU/ml (Fig. 1) as desired, whereas as expected, enterococcal populations were not increased (Fig. 2), confirming that the SC did not contain any Enterococcus spp.

Before inoculation with L. monocytogenes, TGCM samples population of pseudomonads, enterobacteria, and coagulase-positive staphylococci were lower than 2 log CFU/ml, and no Listeria was found in the 25-ml samples (data not shown). L. monocytogenes in inoculated TGCM samples (4.3 ± 0.1 log CFU/ml) was able to increase by ca. 2 log units in the absence of the SC; its growth was more pronounced (P < 0.05) within the first 6 h of incubation at 37 °C (Fig. 3). Mean populations of L. monocytogenes reached a maximum of 6.2 ± 0.4 log CFU/ml after another 6 h of incubation at 18 °C, followed by small (P > 0.05) decreases thereafter. In TGCM+SC samples, the growth pattern of L. monocytogenes was similar; however, its growth within the first 6 to 12 h was reduced. Thus, the presence of the SC in TGCM had a significant growth retarding effect (P < 0.05) on L. monocytogenes (Fig. 3). This effect coincided with faster and greater growth (P < 0.05) of the starter LAB in TGCM+SC samples compared with growth of the natural LAB flora in TGCM samples during incubation at 37 °C for 6 h (Fig. 1). However, the SC proliferation and activity could not completely inhibit the growth of L. monocytogenes in TGCM at 37 °C (Fig. 3), the standard milk curdling temperature in Graviera cheese processing.

The observed growth inhibition of L. monocytogenes at 12 to 72 h of incubation at 18 °C (Fig. 3) was associated with major increases (>8 to 9 log CFU/ml) of LAB populations in both TGCM and TGCM+SC cultures (Fig. 1). However, although in TGCM samples the behavior of LAB populations on M-17 agar (Fig. 1) was similar to that of enterococcal populations on KAA agar (Fig. 2), in TGCM+SC samples major differences between the populations on M-17 and KAA agar plates were observed.
Enterococcal populations in TGCM + SC samples remained ca. 3 log CFU/ml lower ($P < 0.05$) than those in TGCM samples throughout incubation (Fig. 2). Thus, the natural LAB flora in TGCM cultures was dominated by indigenous enterococci, which in TGCM + SC cultures were overgrown by the mixed starter LAB strains. The above major differences in LAB behavior also were reflected in the pH reductions of milk, which were much faster and greater ($P < 0.05$) in TGCM + SC than in TGCM samples (Fig. 4) because of the prevalence of the starter LAB, which had a higher acidification capacity than did the enterococci. In addition to lower pH, TGCM + SC samples had lower populations of $L$. monocytogenes than did TGCM samples during incubation at 18°C; however, this difference was significant ($P < 0.05$) at only 48 h (Fig. 3).

Behavior of $Listeria$ species during Graviera cheese processing and storage. Populations of inoculated non-pathogenic strains of $L$. monocytogenes and $L$. innocua (2.6 ± 0.7 log CFU/g) decreased significantly ($P < 0.05$) in fresh brined cheeses at the onset of ripening, i.e., from day 0 to days 3 and 5 (Table 1). Most of this decrease occurred within the first 24 h and coincided with major increases ($P < 0.05$) in mesophilic and thermophilic LAB populations (Table 1) and a significant decrease ($P < 0.05$) in cheese pH from 6.3 ± 0.2 to 5.9 ± 0.1. After this initial decrease, however, no further significant changes ($P > 0.05$) in $Listeria$ populations occurred during cheese ripening and storage. Clearly, the nonpathogenic indicator strains survived without growth but also without death during the 20-day ripening process and continued to survive with little death in the cheese core during storage at 4°C (Table 1). Long-term survival of these $Listeria$ species occurred despite the fact that LAB populations exceeded 8 log CFU/g in the ripening cheeses. Populations of thermophilic LAB were higher than those of mesophilic LAB from day 1 to day 11, after which there was a reversal trend. Indigenous enterococcal populations progressively increased until midripening (day 11) and then declined slightly (Table 1). The mean (±SD) pH of the cheeses on day 1 (5.9 ± 0.1) decreased further to 5.5 ± 0.1 by day 11 and remained constant ($P > 0.05$) throughout ripening (pH 5.5 ± 0.2) and storage (pH 5.6 ± 0.2). The moisture of the cheeses decreased from 40.5% ± 0.6% before brining (day 1) to

FIGURE 1. Growth of lactic acid bacteria (enumerated on M-17 agar at 30°C) in thermized Graviera cheese milk (TGCM; 63°C for 30 s), with or without a commercial starter culture (SC), that was artificially contaminated with $Listeria$ monocytogenes and incubated for 6 h at 37°C and then for 66 h at 18°C. Each data point represents the mean ± standard deviation.

FIGURE 2. Growth of enterococci (enumerated on KAA agar at 37°C) in thermized Graviera cheese milk (TGCM; 63°C for 30 s), with or without a commercial starter culture (SC), that was artificially contaminated with $Listeria$ monocytogenes and incubated for 6 h at 37°C and then for 66 h at 18°C. Each data point represents the mean ± standard deviation.
31.6% ± 0.8% after ripening (day 23) and to 30.7% ± 1.6% by the end of storage (day 60).

DISCUSSION

A previous validation study conducted in our laboratory provided evidence that fully ripened Greek Graviera cheeses (pH 5.6, aw of 0.948, and 34.8% moisture) that may be surface contaminated with L. monocytogenes postprocessing do not support growth of the pathogen, regardless of packaging (air or vacuum) and storage temperature (4, 12, or 25°C) (15). The present validation study provides further evidence of complete growth inhibition of potential Listeria contaminants in the core of Graviera cheeses throughout processing and storage. In particular, fresh Graviera cheeses traditionally manufactured from thermized milk that may become contaminated during molding, pressing, and/or draining operations will not support Listeria growth. Instead, under the conditions of this study, a majority of the contaminants was inactivated within 24 h after cheese molding and before brining (Table 1). Reductions in Listeria populations occurred despite the fact that the mean pH (5.9) and moisture (40.5%) of the fresh mini cheeses were more supportive of Listeria growth than were these values for the larger, fully ripened Graviera cheeses validated previously (15). Furthermore, surviving Listeria strains were unable to grow after brining and during ripening and storage of the mini cheeses (Table 1), which as expected contained less moisture (ca. 31%) than did the larger cheeses (ca. 35%) (15) when fully ripened. Thus, based on the results from both validation studies, growth of potential Listeria contamination in RTE Graviera cheeses would be suppressed even if the cheeses contained the maximum allowable water content of 40% (2). The observed inability of the L. monocytogenes and L. innocua strains to proliferate in the core of the Graviera cheeses during ripening (Table 1) and in the core (Table 1) or on the surface (15) of fully ripened Graviera cheeses during storage is consistent with previous data on the behavior of L. monocytogenes in other traditional types of hard cheese, such as Colby (38), Cheddar (33), Parmesan (39), and Swiss (3, 7), artificially contaminated with the pathogen during or after processing (14, 32).

Several factors in combination contribute to minimizing contamination and growth of L. monocytogenes and other Listeria species during Graviera cheese manufacture and storage. A primary control factor is milk thermization (60 to
A contamination of thermized milk under 1.86 (0.12) A and spp. during processing and storage L. monocytogenes 7.28 (0.59) Cu

A BC 7.87 (0.18)

Listeria, 8.13 (0.18) Bu

Db in TGCM AB (5, 20) 8.06 (0.27)

7.87 (0.36) BC ALOA (37)

8.12 (0.15) 7.39 (0.54) different (, Listeria contaminants may occur BC z was prohibited. However, the ability of spp. KAA (37)

u CD C Listeria. In contrast, Listeria 7.06 (0.67)

Changes in populations of inoculated nonpathogenic 8.17 (0.25)

8.28 (0.15) L. monocytogenes A

Cheese was manufactured from thermized milk (63
cu BC L. Enterococcus C

7.60 (0.49) A

C Enterococcus durans (16) inoculum was a three-strain cocktail of nonpathogenic Listeria strains.

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TABLE 1. Changes in populations of inoculated nonpathogenic Listeria, mesophilic lactic acid bacteria (LAB), thermophilic LAB, and indigenous Enterococcus spp. during processing and storage of Greek Graviera cheese*  

<table>
<thead>
<tr>
<th>Bacterial type</th>
<th>Plating medium (conditions)</th>
<th>Mean (SD) bacterial population (log CFU/g; n = 6) in cheese on day:</th>
</tr>
</thead>
<tbody>
<tr>
<td>Listeria inoculum*</td>
<td>ALOA (37°C, 48 h)</td>
<td>2.58 (0.65) AB 1.80 (0.24) AB 1.86 (0.12) AB 1.69 (0.37) A 1.68 (0.37) A 1.72 (0.35) A 1.77 (0.23) A 1.44 (0.54) A 1.48 (0.31) A</td>
</tr>
<tr>
<td>Mesophilic LAB</td>
<td>M-17 (22°C, 72 h)</td>
<td>5.82 (0.45) A 7.28 (0.59) B 7.39 (0.54) B 7.87 (0.36) BC 8.28 (0.15) c 8.46 (0.14) c 8.26 (0.32) c 8.13 (0.18) c 7.96 (0.25) BC</td>
</tr>
<tr>
<td>Thermophilic LAB</td>
<td>M-17 (42°C, 48 h)</td>
<td>5.84 (0.65) A 7.87 (0.18) BC 8.12 (0.15) BC 8.17 (0.25) BC 8.46 (0.21) c 8.41 (0.29) c 8.06 (0.27) BC 7.93 (0.27) BC 7.60 (0.49) B</td>
</tr>
<tr>
<td>Enterococcus spp.</td>
<td>KAA (37°C, 48 h)</td>
<td>3.51 (0.42) A 5.21 (0.35) B 5.99 (0.94) BC 7.06 (0.67) CD 8.01 (0.30) d 7.90 (0.25) d 7.69 (0.41) d 7.45 (0.48) d 6.99 (0.75) CD</td>
</tr>
</tbody>
</table>

* Cheese was manufactured from thermized milk (63°C for 30 s) with a product-specific commercial starter culture added.  

b Within the same row, mean values with different letters are significantly different (P < 0.05).  

c Listeria inoculum was a three-strain cocktail of nonpathogenic Listeria strains.
enterococci populations were ca. 1 log lower than those of mesophilic LAB by the end of ripening (Table 1). This starter-dependent difference in prevalence of enterococci in matured Graviera products may have important technological and safety implications, given the controversial role of enterococci in cheese (13, 17, 26). In one study (16), E. faecium was predominant in RTE naturally fermented Graviera cheeses, and several strains were able to produce antilisterial bacteriocins, e.g., enterocins. Genes for variety of bacteriocins consisting mainly of enterocins A, B, P, 50A, and 50B produced by E. faecium and plantaricin A produced by Lactobacillus plantarum were detected in situ by PCR in matured Graviera cheeses manufactured with the commercial SC (15). Thus, another hurdle factor potentially contributing to the observed inhibition of Listeria growth in Graviera cheese might be in situ enterocin production by indigenous E. faecium strains.

In conclusion, Graviera cheeses will not support growth of L. monocytogenes if these cheeses are accidentally contaminated with up to 1,000 CFU/g during manufacture or if they still contain 100 CFU/g or 100 CFU/cm² at the time of sale. However, to assure Listeria inhibition, a starter culture as effective as the mixed LAB starter added to Graviera cheeses in our present and previous (15) validation studies must be utilized routinely in commercial plants. If these starters are used, then RTE Graviera cheeses should be regarded as safe throughout their commercial shelf life even though their usual pH and aw values of 5.6 and 0.95, respectively, are outside the range of the EU specifications (pH ≤ 5.0 and aw ≤ 0.94) for foods regarded as safe from L. monocytogenes contamination. This research-based conclusion is of practical importance because it enables Greek processors to market Graviera cheeses containing L. monocytogenes at up to 100 CFU/g. However, because the pathogen can survive in hard cheeses (3, 32, 37) including Graviera, it is strongly recommended that all necessary process and sanitation measures should be taken in plants to produce cheeses that are free of Listeria at the time of sale. To achieve this goal, supplementation of commercial starters with bioprotective adjunct LAB strains able to enhance in situ inactivation of L. monocytogenes may be feasible (29) if such LAB strain composites are properly optimized under commercial cheese manufacturing conditions.

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