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Comparative qualitative and quantitative analysis of lactic acid bacteria by molecular methods in different Greek cheeses

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Research Article

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Abstract

In the present research communication, we report on identification and quantification of four main lactic acid bacteria (LAB) genera (Lactococcus, Lactobacillus, Streptococcus and Leuconostoc), most common in Greek cheeses, by a novel culture-independent method. More specifically, new primers were designed to be used in both multiplex PCR for simultaneous identification and in real-time PCR for quantification of the LAB. The method was validated by applying it in parallel to culture-dependent method in a variety of cheeses from different Greek geographical locations, of different animal milk origins and of different production methods. While the standard plate culture method showed absence of *Leuconostoc* sp. in all cheeses, the culture-independent methods detected all four LAB genera studied. Furthermore, the relative presence of the four genera detected by the culture-independent method showed a pattern present in almost all cheese samples tested, indicating *Lactococcus* genus as the dominant one.

The microbiota of dairy products consists of, apart from starter and non-starter lactic acid bacteria, yeasts and filamentous fungi that form secondary microbiota with a significant role during cheese ripening (Beresford *et al.*, 2001). LAB used as starter cultures in cheese production are essential for the development of organoleptic characteristics. Starter and secondary bacteria modify the physical and chemical properties of cheese and largely influence its characteristics (Steele *et al.*, 2012; Gobbetti *et al.*, 2015).

Up to recent years, the study of microbial community of cheeses was performed mainly by a conventional plate counting method, which has certain drawbacks such as high time consumption and often uncertainty owing to an increasing number of species that vary in only a few characteristics (Requena and Peláez, 2010). In the last two decades, however, molecular methods have attracted the interest of scientists as culture-independent tools, because of certain advantages such as simplicity, repeatability, specificity and relatively low cost. However, some of them especially metagenomic analysis by next-generation sequencing (NGS) are still quite costly and the identification at species level is still not very satisfactory. Nevertheless, the choice of the appropriate method is still a requisite and quite often polyphasic studies, including both culture-dependent and culture-independent approaches are applied (Alegria *et al.*, 2012).

In Greece, cheeses are usually produced from pasteurized milk with the addition of either industrial or artisan starters and most cheeses are hard. Almost every Greek region produces a unique type of cheese. In Greek islands, due to the characteristic microclimate, soil, other environmental factors and technology, very special types of cheese are produced (Litopoulou-Tzanetaki and Tzanetakis, 2011).

In our laboratory we designed new primers to be used for a novel multiplex PCR and a novel real-time PCR as culture-independent methods for the identification and quantification of the LAB present in cheese. We applied these methods for analyzing a total of thirty-six Greek cheeses in terms of qualitative determination of the four representatives LAB genera (*Lactobacillus*, *Lactococcus*, *Streptococcus*, *Leuconostoc*) most frequently found in Greek cheeses. Of these samples, all were tested with the multiplex PCR. All these cheeses have also been tested for the animal origin of the milk by a specific molecular method according to Tsirigoti *et al.* (2020). Nine of these cheeses, which were fully analyzed regarding the milk origin by molecular protocols, were chosen as representatives from different Greek regions and were further analyzed by both the conventional culture plate method and the novel real-time PCR.

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Materials and methods

Sampling

Thirty-six different local cheeses from dairies of the Greek mainland (10) and from different Greek islands (26) were analyzed. Eleven were produced from pure cows' milk, three from sheep's milk, six from goat's milk and the rest from a mixture of cow, goat and sheep's milk (ten), goat and sheep's milk (five) and finally cow and sheep's milk (one). Twenty-three of them were classified as hard cheese, nine as semi hard and four as soft. The nine cheeses chosen for further analysis by a combination of culture methods and real-time PCR, were of cow's milk (2), goat's milk (2), sheep's milk (1), goat and sheep's milk (2) and cow, goat, and sheep's milk (2), whereas according to cheese type, six were hard, two semi hard and one soft.

DNA extraction

DNA was extracted out of 10 g of homogenized cheese according to methods described in detail in the online Supplementary File, and using the Pure Link Genomic DNA Extraction Kit (Life Sciences-Thermo Fisher Scientific, USA) from Gram positive bacteria.

Qualitative determination of the four LAB genera by multiplex PCR

Specific fragments of *tuf*, *hrcA* and *lacZ* genes were chosen for the identification of the genera *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp. and *Streptococcus* sp. Five primer pairs were designed, to amplify different size fragments of these genes suitable for both multiplex PCR and real-time PCR (Table 1).

Genomic DNA of reference bacterial strains was used to test the specificity of the multiplex PCR. Multiplex PCR reactions were performed according to the method described in the online Supplementary File.

Quantitative determination of the four LAB genera

For the quantitative determination we applied both the plate culture method (Cabezas *et al.*, 2007) and the real-time PCR as described in the online Supplementary File.

Statistics

The R programming (v 3.5.1) language was used for statistical computing and graphics. The significant statistical differences were determined by Welch t-test with significance declared as $P < 0.05$.

Results

Qualitative determination of lactic acid bacteria

The specificity of the multiplex PCR was tested by a number of reference bacterial strains (described in the online Supplementary File). In 35 out of 36 cheeses tested, all four genera were detected by the novel multiplex PCR (online Supplementary File, Fig. S1). In one semi-hard cheese sample, produced by cow milk originated from the mainland, the *Lactobacillus sp.* was poorly detected.

Quantitative determination of lactic acid bacteria

Nine out of the thirty-six analyzed cheese samples were further analyzed as representatives from different Greek regions by both the conventional culture plate method and the novel real-time PCR. These nine cheeses were all tested by the specific triplex-PCR on somatic cell DNA, described by Tsirigoti *et al.* (2020) that enables the identification of the species of origin (cow, sheep, goat), and all were shown to be correct as labelled.

The population of the four genera of LAB counted by real-time PCR are shown in a logarithmic scale in Figure 1. In almost all the cheese samples tested, a pattern of the relative population of the LAB was repeated independently of the milk originality (ie cow, goat, sheep, or a mix of them) (Fig. 1a), the type of cheese (hard, semi hard, soft) (Fig. 1c) and the geographical location (data not shown). *Lactococcus* sp was dominant, at a significant level ($P < 0.05$), against the rest of the genera studied. No statistically significant differences among the remaining genera were noticed.

As shown in Figs. 1b and 1d the application of the standard plate culture method detected *Lactobacillus*, *Lactococcus* and *Streptococcus* genera in significant populations. *Leuconostoc* genus was not detected in any of the tested cheeses. No statistical

Table 1. Primers used in Novel multiplex and Real Time PCR Sequences of the primers designed for the four LAB genera and the size of the amplified fragment

Primer	Genus – target	Sequence 5′→3′	Position	Length
Leuc F	*Leuconostoc*	ACT TAC GTT CGC ACT AAG CCC	Tuf gene, NC_008531.1 13–33 nt&548–569 nt	557 bp
Leuc R		TCG ATA ACC TTA ACT TGT TCA G		
StrTherm F	*Streptococcus*	CAA GGG GTT GCT ACT TCC ATC	lacZ gene, NC_006448.1 430–450nt &868–887 nt	457 bp
StrTherm R		GGA CTT TCA GCA CTC CAA GG		
Lactu F	*Lactococcus*	GAA CGG TGA ACC ACA ATG GGT	Tuf gene, NC_002662.1 537–560 nt&902–923 nt	386 bp
Lactu R		CCT TGG AAA AGT TTG TGT GGA G		
LachrcA F	*Lactobacillus*	GCC TAA TTT TAA AGG CCA TTG TCC	hrcA, NC_JN620373.1 81–104 nt&379–401 nt	320 bp
LachrcA R		AGA TAT CCG CCG ACT GAG CAA CG		
Lball F	*Lactobacillus*	GTC ATG GCC ATT TTG GTC ACG	hrcA, N ACGY01000129.1 439–459 nt&688–709 nt	270 bp
Lball R		ACC GCC CGC CAA CAT AAA AAC G		

The pair of primers LachrcA F/R detects the Lactobacillus sp (specifically Lactobacillus pentosus, Lactobacillus plantarum and Lactobacillus paraplantarum), while the pair Lball F/R detects the species Lactobacillus casei, Lactobacillus paracasei and Lactobacillus brevis.

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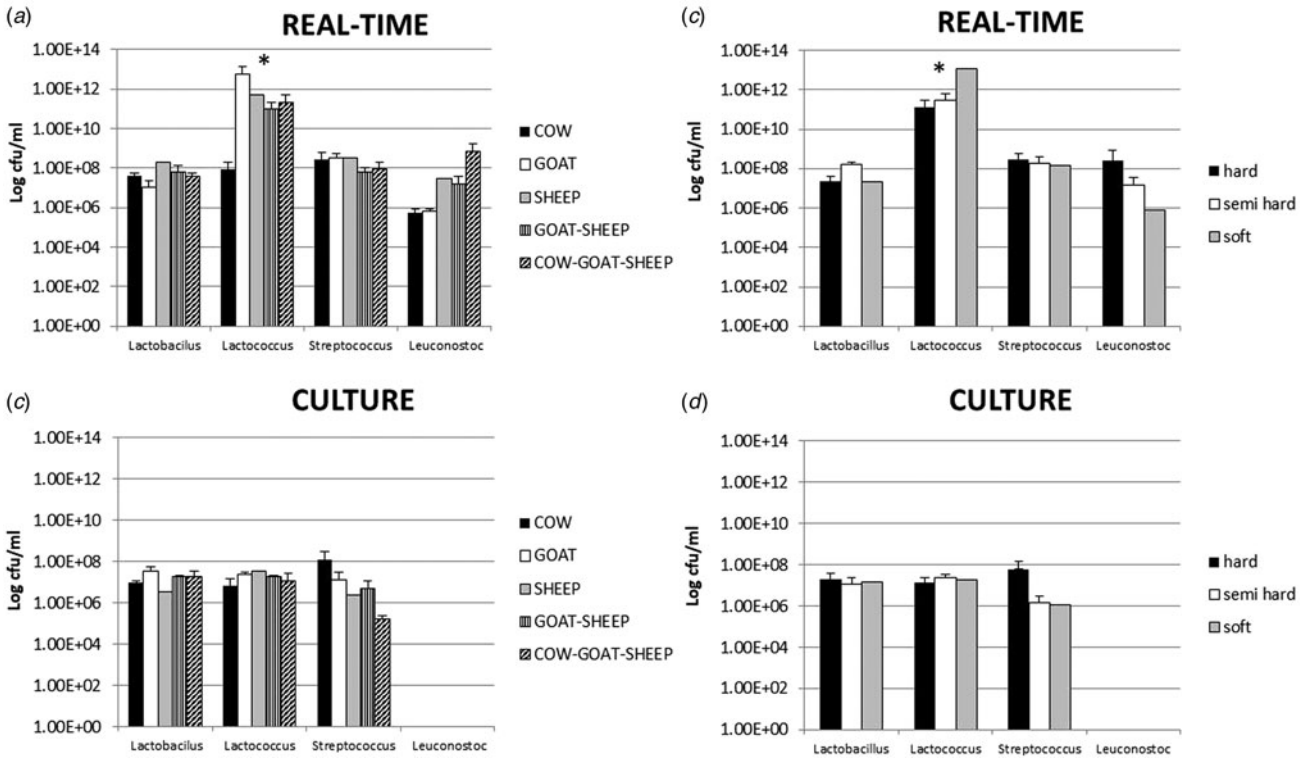


Fig. 1. Population of the four LAB genera determined by real-time PCR (copy numbers: 1a and 1c) and Standard Plate Method (CFU/ml: 1b and 1d). Data are presented in logarithmic scale. The cheese samples were separated into five groups of cheeses, (according to the animal origin of milk: 1a, 1b, or the cheese type: 1c, 1d).

differences were found among the three genera (*Lactobacillus*, *Lactococcus* and *Streptococcus)* of the cultured LAB.

Discussion

The role of the four main bacteria genera used in almost all the cheese production methods for the starter culture, (*Lactobacillus*, *Lactococcus*, *Streptococcus* and *Leuconostoc*) is extremely important (Beresford *et al*., 2001). According to many reports *Lactobacillus* dominates cheese ripening (Gurses and Erdogan, 2006), although the actual effect of the milk origin, the geographical location and the production technology of different types of cheese (hard, semi hard, soft) is not yet fully understood. Successful identification, typing and characterization of the microorganisms in cheese microbiota and enumeration of their actual populations are essential (Pogacic *et al*., 2010). In the present research communication, we report on an approach to the above-mentioned questions by a combination of culture plate dependent and novel culture-independent methods.

Specific primers were designed in our laboratory targeting the four essential and most common genera of LAB in Greek cheeses. They were designed to be used in both a novel multiplex PCR for the qualitative analysis and in a novel real-time PCR for quantitative analysis in cheeses. The specificity of the novel multiplex PCR was confirmed by applying the method on the reference bacterial strains. The application of multiplex PCR clearly indicated the presence of all four genera of LAB in 35 out of the 36 cheeses tested. This was expected since all the cheeses originated from pasteurized milk and their microbial community is determined mainly by the starter cultures (Gobbetti *et al*., 2015).

The novel real-time PCR offered quantitative determination for all four LAB genera. The population of *Lactococcus* was consistently higher than the remaining genera with the only exception observed in cheeses produced from cow's milk. In these cheeses we also observed *Streptococcus* presenting a slightly increased population from the remaining genera. Domination of *Streptococcus* and *Lactococcus* species in cow's milk and *Lactococcus* in goat's milk cheeses has been reported by Zhu *et al*. (2020) in China. According to our results it seems that the bacterial community in Greek cheeses is not affected by the geographical location of the producing place, the type of cheese and to a lesser extent the origin of milk.

Comparing the results of the culture-independent method with the standard plate count method, the former has proven to be more accurate and valuable. The culture dependent method did not exhibit statistically significant differences in the CFU among the LAB genera and failed to detect any CFU of *Leuconostoc sp*. in any of the analyzed samples. It also gave significantly lower counts for *Lactococcus*, which may be attributed to the fact that real-time PCR detects the *Lactococcus* DNA present in the milk used for the production and through the whole ripening process of cheeses samples, whether they are alive or not. Many research reports mention an increased population of *Lactococcus sp*. that does not survive up to the end of the ripening process (Dolci *et al*., 2008; Litopoulou-Tzanetaki and Tzanetakis, 2014). *Lactococcus* is sensitive to low pH and high NaCl concentration (Rantsiou *et al*., 2004). Furthermore, carbohydrate starvation may force *Lactococcus* to a nonculturable state although they remain alive for at least another two weeks (Ganesan *et al*., 2007).

As far as the genus *Leuconostoc* is concerned, the observed absence of living bacteria, indicated by the standard culture method, is not an unusual phenomenon. These results were in agreement with Cogan *et al*. (1997), who found that five cheeses,

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amongst them three of Greek origin (Kasseri, Feta, fresh cheese), did not present bacteria of this genus. However, application of real-time and multiplex PCR, indicated the presence of significant levels of DNA of *Leuconostoc* sp. in the tested samples which is in line with many previous reports according to which *Leuconostoc* represents a dominant genus in the process of cheese production (Friedrich and Lenke, 2006). *Leuconostoc* species are known for their role in flavor and aroma of cheese, and are often found in significant populations, especially at the beginning of ripening (Gerasi *et al.*, 2003) but a lot of studies have demonstrated a significant decrease or even absence at the end of ripening (Litopoulou-Tzanetaki and Tzanetakis, 2014). Also, other comparative studies of culture-independent methods such as real-time PCR and FLOW-FISH, indicated that viable bacteria are present in cheese but are not able to be cultured (Friedrich and Lenke, 2006; Ganesan *et al.*, 2007).

In conclusion, our results point to a relatively low cost and reliable molecular method for LAB identification and quantification in cheese samples. Inconsistencies between this method and the plate count method are probably due to the lower sensitivity of the latter. The results also showed a rather persistent pattern of the four LAB genera in all Greek cheeses with *Lactococcus sp.* being the most abundant and this pattern is independent of geographical location, milk-producing animal species and type of cheese.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S0022029922000760

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