COMPARATIVE QUALITATIVE AND QUANTITATIVE ANALYSIS OF LACTIC ACID BACTERIA BY MOLECULAR METHODS IN DIFFERENT GREEK CHEESES

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SUPPLEMENTARY FILE

METHODOLOGY

Qualitative determination of the four LAB genera by Multiplex PCR

Specific genes' fragments were chosen for the identification of the genera *Lactobacillus* sp., *Lactococcus* sp., *Leuconostoc* sp. and *Streptococcus* sp. which are the most common genera found in Greek cheeses.

Genomic DNA of the reference bacterial strains *Lactococcus lactis* ssp. *lactis* ATCC 19435, *Lactococcus garviae* 0232, *Lactococcus lactis* ssp. *cremoris* 0075, *Lactobacillus plantarum* ATCC 14917, *Lactobacillus plantarum* ATCC 10012, *Lactobacillus paracasei* ssp. *paracasei* ATCC 25302, *Lactobacillus pentosus* NCFB 363, *Leuconostoc mesenteroides* ssp. *mesenteroides* ATCC 8293, *Leuconostoc mesenteroides* ssp. *mesenteroides* LMG 6893, *Streptococcus thermophilus* LMG 13565, *Streptococcus macedonicus* 210, *Enterococcus faecalis* ATCC 19433, *Pseudomonas aeruginosa* NCIMB 12469, *Bacillus megaterium* ATCC 11478, *Staphylococcus aureus* NCIMB 8625 and *Shigella flexneri* ATCC 29903, was used in order to test the specificity of the multiplex PCR.

Multiplex PCR reactions were performed in a final volume of 10 μl containing 1x KAPA 2G Multiplex PCR Mix (KAPA Biosystems), 300nM of each primer and 80-100ngDNA.The

protocol included an initial denaturation step for 3 min at 95°C, followed by 30 cycles of three steps: denaturation at 95°C for 15 sec, annealing of the primers at 60°C for 30 sec and elongation at 72°C for 30 sec. The final elongation takes place at 72°C for 7 min.

Quantitative determination of the four LAB genera

For the quantitative determination were applied both, the plate culture method, and the Real-Time PCR

Standard plate culture method

Each sample was homogenized with 0.09% NaCl, and 100 μl of subsequent dilutions were inoculated in appropriate growth media, (MRS, M17, Todd-Hewitt and MRS supplemented with vancomycin was used for *Lactobacillus* sp., *Lactococcus* sp., *Streptococcus* sp. and *Leuconostoc* sp., respectively). After 24 to 48 hours of incubation at 30^{o}C for *Leuconostoc* sp. and *Lactococcus*sp. and 37^{o}C for *Lactobacillus* sp. and *Streptococcus* sp., strains of different distinct colonies were characterized as bacillus, coccus or coccobacillus by Gram staining. A test with inverted Durham tube in order to check the production of CO_2 (incubation at 30^{o}C in MRS broth) was conducted especially for the bacteria which were growing in MRS substrate with vancomycin and were characterized as coccobacillus in addition to catalase and peroxidase tests were carried out. The appropriate colonies (according to morphological and biochemical criteria) were counted and the cfu/ml were calculated. Each sample was analyzed twice.

Real-Time PCR

Real-Time PCR was performed using the same primers as the multiplex PCR (Table 1), in a final volume of 20 μl including 1x Platinum SYBR Green qPCRsupermix-UDG (Thermo Fisher Scientific), 300nM of each primer and 40ng DNA.

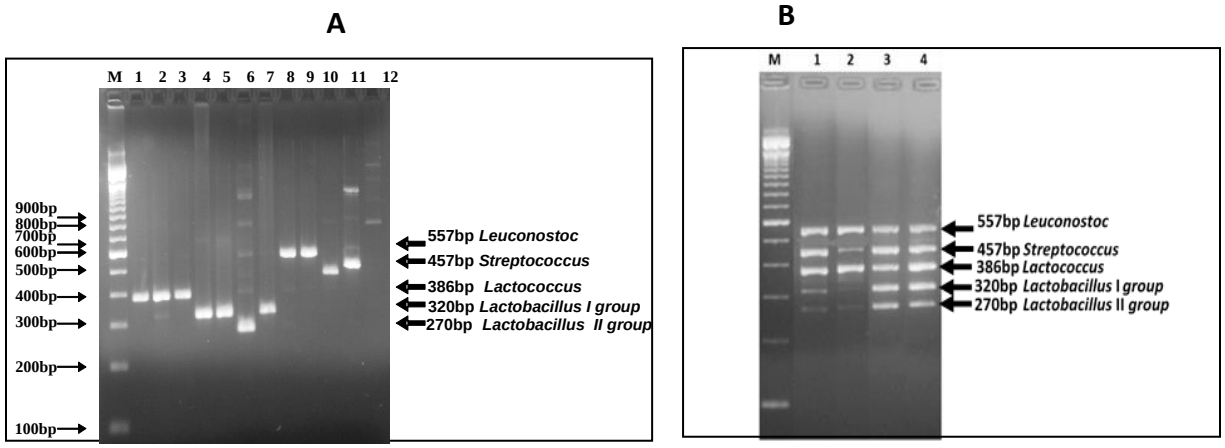
The DNA fragments were amplified in Chromo4™ Four-Color Real-Time Detector (Bio-Rad) with the following conditions: activation of UDG at 50°C for 2 min and initial denaturation at 95°C for 2 min, followed by 40 cycles of two steps: denaturation at 95°C for 15 sec and annealing / elongation at 60°C for 30 sec. All reactions were performed in triplicates and checked by agarose gel electrophoresis. No-template control reactions were also included for each target gene.

Standard curves were generated by plotting the cycle threshold values (Ct) of the amplified genes against the $\log_{10}$ of the bacterial copies which were calculated by using DNA extracted from the tenfold dilution of the initial cultures. Each standard curve was performed at least three times. The reference bacterial strains used for the standard curves were *Lactobacillus paracasei* subsp. *paracasei* ATCC 25302, *Lactobacillus plantarum* 14917, *Lactococcus lactis* subsp. *lactis* 20481, *Streptococcus thermophilus* LMG 13565 and *Leuconstoc mesenteroides* 6893. The detection limit (cfu/ml) of cells from each standard bacterial strain used for the generation of standard curves in Real-Time PCR and the coefficient of determination (R^2) are shown in Table S1.

Table S1. Detection limit (cfu/ml) and the coefficient of determination (R^2) of the reference bacterial strains used as basis for the generation of standard curves used in Real-Time PCR.

Reference strains	**cfu/ml**	**R^2**
Lactobacillus paracasei subsp. *paracasei* ATCC 25302	$7.50x10^9$ - $7.50\ x10^2$	1
Lactobacillus plantarum 14917	$4.00x10^{10}$- $4.00\ x10^2$	0.999
Lactococcus lactis subsp. *lactis* 20481	$2.33x10^{10}$ - $4.00\ x10^2$	0.971
Streptococcus thermophilus LMG 13565	$1.72x10^9$ – $1.72\ x10^2$	0.975
Leuconstoc mesenteroides 6893	$3.67x10^{11}$ - $3.67x10^2$	0.967

Figure S1. Validation of novel Multiplex PCR specificity and qualitative determination of LABs in cheese samples



A: Electrophoresis on 2.5% Agarose gel of the amplicons of the novel multiplex PCR method on 12 reference bacteria strains. M: 100bp DNA ladder (Invitrogen). 1: *Lactococcus lactis ssp. lactis* ATCC 19435, 2: *Lactococcus garviae* 0232, 3: *Lactococcus lactis ssp. cremoris* 0075, 4: *Lactobacillus plantarum* ATCC 14917, 5: *Lactobacillus plantarum* ATCC 10012, 6: *Lactobacillus paracasei ssp. paracasei* ATCC 25302, 7: *Lactobacillus pentosus* NCFB 363, 8: *Leuconostoc mesenterooides ssp. mesenteroides* ATCC 8293, 9: *Leuconostoc mesenteroides ssp. mesenteroides* LMG 6893, 10: *Streptococcus thermophilus* LMG 13565, 11: *Streptococcus macedonicus* 210, 12: *Enterococcus faecalis* ATCC 19433.

B. Qualitative determination of LAB in cheese samples. Electrophoresis from multiplex PCR products on 2.5% Agarose gel. M: 100bp DNA ladder (Invitrogen), 1-4: cheese samples containing *Leuconostoc* sp. (557 bp), *Streptococcus* sp. (457 bp), *Lactococcus* sp. (386 bp) and *Lactobacillus* sp. (group I and group II: 320 bp and 270bp, respectively). 1: semi-hard cheese from sheep milk, originated from an island, 2: semi-hard cheese from cow milk, originated from

mainland, 3: hard cheese from goat milk, originated from an island, 4: semi-hard cheese from a mixture of goat, sheep and cow milk, originated from mainland.