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Culture-Independent Quantitative Approach to Monitoring the Dynamics of Bacterial Population During Istrian Cheese Ripening

Mirna Mrkonjić Fuka[1,2]*, *Marion Engel*[2], *Andrea Skelin*[1],
Bojana Bogovič Matijašić[3], *Sulejman Redžepović*[1] and *Michael Schloter*[2]

[1]Department of Microbiology, Faculty of Agriculture, University of Zagreb, Svetošimunska 25,
HR-10000 Zagreb, Croatia

[2]Research Unit for Environmental Genomics, Helmholtz Centre Munich, Ingolstaedter Landstrasse 1,
DE-85764 Neuherberg, Germany

[3]Department of Animal Science, Biotechnical Faculty, University of Ljubljana, Groblje 3,
SI-1230 Domžale, Slovenia

Summary

In order to preserve the specificity of artisanal cheese and to minimize variations in quality, real-time PCR can be applied to monitor the dynamics of autochthonous bacterial population throughout ripening. This may give the basis for the selection of species and strains that can be used to deliver safe products with balanced texture and flavour, and moreover, it can be applied to monitor the abundance of slow-growing or nonculturable species. The aim of this study is to evaluate the application of real-time PCR and plate count analysis in order to follow the dynamics of lactic acid bacteria (LAB) and enterobacteria during the ripening of traditional Istrian cheese. The abundance of all LAB was increased by prolonging the ripening time and reached the plateau after 90 days. The present study demonstrated that *Lactococcus* counts were closest to total bacterial count irrespective of the applied method, confirming *Lactococcus* spp. as one of the dominant bacterial groups associated with the ripening of Istrian cheese. Enterobacteria were mainly present at early phases of cheese ripening, whereas at later time a decrease was visible in samples from all farms.

Key words: real-time PCR, Istrian cheese, lactic acid bacteria, *Enterobacteriaceae*

Introduction

The interactions of microbiota contribute greatly to the cheese ripening process and can be considered as crucial for the cheese character and flavour. Mainly lactobacilli, lactococci and enterococci, which belong to lactic acid bacteria (LAB), contribute significantly to the development of the specific aromatic characteristics of traditional cheese (*1*). They influence cheese ripening by the production of lactic acid, by a decrease in the oxidation-reduction potential, and by the release of proteolytic and lipolytic enzymes (*2*), and are involved in the preservation of food by antagonistic activities against food spoilage microorganisms (*3*). LAB represent up to 30 % of total bacterial counts in raw milk. According to Medina *et al.* (*4*), *Enterococcus* spp. are the majority of LAB in ewe's raw milk (48 %), followed by *Lactobacillus* spp. (30 %), *Lactococcus* spp. (14 %) and *Leuconostoc* spp. (8 %), but their number is variable based on the conditions of production, season, breeding and the origin of milk (*5*). In

contrast to the LAB, which are of high importance for the quality of the cheese, the presence of enterobacteria can jeopardize the quality of dairy products and has been considered as disputable (6,7). Enterobacteria are commonly found in raw milk cheese (8) and some authors suggest that these microorganisms contribute to the cheese taste and flavour (9). However, their presence in high number tends to reflect the poor standards of hygiene during milking and handling, and warns of the possible presence of more dangerous enteric pathogens (10).

Istrian cheese is a traditional dairy product in Croatia that is mainly produced by local farmers on a small scale. Methods and tools of cheese-making are artisanal and traditional; all the phases of manufacture are manual. Consequently, the cheese quality and hygienic procedure are very variable. The cheese is made from raw ewe's milk without the application of starter cultures, thus allowing for spontaneous fermentation. The dry cheese is left to ripen for 90–120 days and has a hard texture and strong flavour (11).

In a previous work we described shifts in the diversity pattern of LAB and enterobacteria by molecular fingerprinting (11). However, quantitative data on the abundance of the two groups of microbes during cheese ripening, which is of high importance to control the ripening process and to set standards for microbiological food safety, are still missing. Molecular analyses have shown that the complexity of microbial communities involved in the fermentation process is much greater than previously thought and that the biodiversity of different food ecosystems is still unknown (12). This lack of knowledge is mostly attributed to the failure of many bacteria to grow in a given culture medium (13). Therefore, we used specific sets of primers for the different LAB groups as well as enterobacteria, which target variable regions of the 16S rRNA gene, and quantitative real-time PCR (qPCR) to characterize the shifts of these organisms during cheese ripening. In addition, we also estimated total bacterial abundance based on universal primers for the 16S rRNA gene and qPCR. However, the use of PCR--based methods can also result in highly biased data, *e.g.* due to the non-selectivity of the used primers for PCR amplification. Thus, it was of high importance to carefully evaluate the specificity of the used primer sets using *in silico* approaches before use in qPCR and to compare CFU data (this and previous study (11)) with qPCR data using correlative analysis.

Materials and Methods

Raw milk and cheese sampling

Four batches of Istrian cheese were manufactured by four cheese makers (F1, F2, F3 and F4) located on the Istrian peninsula (Croatia). The cheese samples were produced from raw, full cream milk from the autochthonous Istrian sheep breed in accordance with traditional methods (11). All the farms are part of a consortium for the preservation of the certified product Istrian cheese and are located in different areas of Istria with different microclimatic conditions, relief and vegetation. Due to the earlier beginning of the production of Istrian cheese at F1 and F2 farms, the collection of samples started at

F1 and F2 farms in May 2007 and at F3 and F4 farms in June 2007. Milk and cheese samples were collected from four individual batches as follows: milk (M0) and fresh cheese (Ch0) were taken at day zero, and cheese samples were sampled after 30 (Ch30), 60 (Ch60), 90 (Ch90) and 120 (Ch120) days. The pH of cheese samples was between 4.8 and 5.2, fat content between 38 and 53 % and salt content between 2.3 and 4.6 %.

Enumeration of different bacterial groups

The milk and cheese samples were submitted to microbiological analysis for enumeration and isolation of *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Enterobacteriaceae* isolates. The cheese samples were homogenized with a Stomacher (BagMixer® 400, Interscience, St Nom, France) for 3 min and diluted tenfold in a sterile physiological solution (0.9 % NaCl). By using the pour plate technique, aliquots of the samples were inoculated on two selective agar plates and incubated for 48 h. Enterococci were grown on citrate azide Tween carbonate agar (CATC, Merck, Darmstadt, Germany) under aerobic conditions at 37 °C, lactobacilli on de Man-Rogosa-Sharpe agar (Oxoid, Basingstoke, UK) at 37 °C under anaerobic conditions, lactococci were grown aerobically on M17 agar (Oxoid) at 30 °C, and enterobacteria were analysed on violet red bile agar (VRBA, Oxoid) at 37 °C under aerobic conditions as described by Randazzo *et al.* (1).

Standard preparation for quantitative real-time PCR (qPCR)

Lactobacillus casei DSM 20011, *Enterococcus faecalis* DSM 20478, *Escherichia coli* DSM 30083T and *Lactococcus lactis* ssp. *lactis* DSM 20481 were purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) and were used for generating standards for qPCR. *Lactobacillus casei* was cultured in MRS medium (Oxoid), *Enterococcus faecalis* in brain heart infusion medium (Merck), *Escherichia coli* in nutrient broth medium (Merck) and *Lactococcus lactis* ssp. *lactis* in M17 (Merck) as recommended by DSMZ. Genomic DNA of bacterial cultures was extracted using DNeasy Tissue Kit (Qiagen, Hilden, Germany) as recommended by the manufacturer.

For the preparation of plasmid standards, DNA from pure cultures was amplified by PCR as described by Byun *et al.* (14), Castillo *et al.* (15), He and Jiang (16), Monnet *et al.* (17) and Bach *et al.* (18). Primer sequences and references are listed in Table 1. The obtained PCR products were purified using a minElute Gel Extraction Kit (Qiagen), cloned into the pDrive Cloning Vector (Qiagen) and transformed into QIAGEN EZ Competent Cells. A Qiagen Plasmid Midi Kit was used to purify plasmid DNA. The plasmid DNA inserts were sequenced to confirm the origin. Quantification of the plasmid DNA was done by NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Wilmington, MA, USA). The DNA for standard curves was stored at –20 °C in aliquots until used in triplicates as external standards for the quantitative PCR.

Table 1. Oligonucleotides used as primers and TaqMan probe for the quantification of 16S rRNA genes

Primer or probe	Composition	Source	Standard origin and target group
LactoF	5′-TGGAAACAGRTGCTAATACCG-3′	(14)	*Lactobacillus casei* (lactobacilli)
LactoR	5′-GTCCATTGTGGAAGATTCCC-3′		
ECF	5′-AGAAATTCCAAACGAATTG-3′	(16)	*Enterococcus faecalis* (enterococci)
ECR	5′-CAGTGCTCTACCTCCATCATT-3′		
ECP	FAM-TGGTTCTCTCCGAAATAGCTTTAGGGCTA-TAMRA		
F-ent	5′-ATGGCTGTCGTCAGCTCGT-3′	(15)	*Escherichia coli* (enterobacteria)
R-ent	5′-CCTACTTCTTTTGCAACCCACTC-3′		
F-Lcocci	5′-GCTCACCAAGGCGATGATACATA-3′	(17)	*Lactococcus lactis* ssp. *lactis* (lactococci)
R-Lcocci	5′-ACCAACGTTCTTCTCTACCAACA-3′		
FP 16S rRNA	5′-GGTAGTCYAYGCMSTAAACG-3′	(18)	*Escherichia coli* (total bacteria)
16S rRNA probe	FAM-TKCGCGTTGCDTCGAATTAAWCCAC-TAMRA		
RP 16SrRNA	5′-GACARCCATGCASCACCTG-3′		

DNA extraction from cheese samples

Total DNA was extracted and purified from 10 mL of milk or 10 g of cheese after homogenization in a sterile physiological solution using a Stomacher (BagMixer® 400) for 3 min at 230 rpm. Prior to the DNA extraction, 10 mL of homogenates were centrifuged at 3500×*g* for 10 min. The supernatant was discarded and the fat layer, which was on top of the liquid, was mechanically removed by a sterile filter paper. DNA was extracted by Maxwell Tissue DNA Purification Kit (Promega, Madison, WI, USA) as described by Mrkonjić Fuka *et al.* (*11*). The quality and quantity of the DNA was estimated by NanoDrop (Thermo Fisher Scientific) and checked by using electrophoresis on 2 % agarose gel.

Verification of primer specificity

The specificity of primer pairs used for real-time PCR analysis was proven *in vitro* (*14–17*). However, to check the primer specificity in a broader bacterial range, an *in silico* proof of primer specificity was done. A virtual PCR using the ModelInspector tool of the Genomatix software package (Genomatix Software GmbH, Munich, Germany) against the data set of bacteria (GenBank Release 166, National Center for Biotechnology Information (NCBI), Bethesda, MD, USA) was performed allowing two mismatches.

Real-time qPCR setup

Real-time qPCR was applied to quantify bacteria belonging to *Enterococcus*, *Lactococcus*, *Lactobacillus* and *Enterobacteriaceae* as well as total bacteria in milk and cheese samples during the ripening from four different farms. Amplification and monitoring were carried out with the ABI 7700 Sequence Detection System (PerkinElmer Inc., Norwalk, CT, USA) using SybrGreen for quantification of lactobacilli, enterobacteria and lactococci, and a TaqMan probe (the corresponding sequences are listed in Table 1) as the detection system for the quantification of total bacteria and enterococci. For the real-time PCR assay the same primer sets and conditions were used as for the preparation of plasmid standards.

In order to eliminate the inhibition of PCR, milk and cheese DNA from all four farms were serially diluted at a ratio between 1:5 and 1:100. Dilution ratio that gave the lowest cycle threshold (Ct) value was further used for the real-time PCR analysis of the targeting bacterial group. The standard curves were created using a 10-fold dilution series of standard DNA. Specificity of the real-time PCR assay was checked by both melting curve analysis after each real-time PCR run, and on agarose gel where 10 % of real-time PCR products from one of three independent replicates of each of the dairy samples were loaded and electrophoretically separated. Since the number of 16S rRNA genes per genome differs in prokaryotic organisms (*19*) and is highly variable between even closely related LAB or enterobacteria, the number of each of the targeting bacterial populations was calculated as gene copy number per 1 g of cheese or 1 mL of milk and not transformed into the number of organisms.

Statistical analysis

All statistical analyses were performed using R project software (*20*) and SPSS statistical program (SPSS, v. 12.0, Munich, Germany). Effects of ripening time and farm conditions as well as their interactions on cheese variables were tested using ANOVA and univariate analysis of variance on log-transformed data. The post hoc Tukey-Kramer test was used to determine the differences between ripening mean values ($p<0.05$) or farm mean values ($p<0.05$). Correlation between variables (CFU and real-time PCR data) for each bacterial group was tested by Pearson's product-moment correlation. Friedman test was used to test for significant differences between CFU bacterial counts or between real-time PCR data.

Results

Enumeration of bacteria using culture-dependent approaches

The dynamics of microbial counts during Istrian cheese ripening is shown in Table 2. The counts of all in-

Table 2. The mean number of lactococci, enterococci, lactobacilli, enterobacteria and total bacterial count obtained by real-time PCR and agar viable count

| Sample | Lactococci | | Enterococci | | Lactobacilli | | Enterobacteria | | Total bacteria count |
	M17 *a***	real-time PCR *A***	CATC *a***	real-time PCR *B***	MRS *b***	real-time PCR *B***	VRBA *c***	real-time PCR *B***	real-time PCR
Farm	ns	ns	ns	*	ns	ns	ns	*	*
F1	6.67±0.90	7.24±0.84	6.91±1.21	(7.62±1.28)^b	5.75±1.53	6.74±1.26	3.14±1.84	(6.77±1.40)^{ab}	(8.30±1.06)^a
F2	6.43±0.65	7.52±1.36	5.91±1.12	(6.75±1.02)^{ab}	5.45±1.51	6.46±1.35	3.44±1.94	(7.53±0.55)^b	(9.03±0.45)^b
F3	6.29±0.68	7.64±1.25	5.95±1.62	(5.60±1.81)^a	5.28±1.86	5.98±1.78	3.67±2.05	(6.48±1.19)^a	(9.05±0.77)^b
F4	6.88±0.55	7.21±1.42	6.19±0.79	(6.62±1.21)^{ab}	5.53±1.74	6.41±1.35	2.30±1.96	(6.30±1.11)^a	(8.93±0.79)^b
Time/day	*	*	*	*	*	*	*	*	*
M0	(5.77±0.40)^a	(5.60±0.72)^a	(4.22±0.97)^a	(4.36±1.55)^a	(2.51±1.54)^a	(3.99±1.00)^a	(2.71±1.55)^b	(5.64±1.58)^a	(7.76±0.80)^a
Ch0	(6.11±0.71)^b	(6.50±0.79)^b	(5.76±1.10)^b	(6.08±1.19)^b	(4.83±0.98)^b	(5.46±1.09)^b	(3.60±1.31)^b	(6.87±0.82)^{bc}	(8.35±0.62)^b
Ch30	(7.00±0.39)^b	(8.11±0.75)^{cd}	(6.76±0.65)^c	(7.15±0.55)^{bc}	(6.44±0.34)^c	(6.86±0.42)^c	(5.30±0.75)^c	(8.05±0.29)^d	(9.41±0.66)^c
Ch60	(6.72±0.42)^b	(8.05±0.64)^{cd}	(6.88±0.69)^c	(7.01±0.87)^c	(6.63±0.28)^{cd}	(7.11±0.42)^c	(3.72±0.74)^b	(7.13±0.98)^c	(8.91±0.58)^c
Ch90	(6.78±0.63)^b	(8.47±0.29)^d	(6.92±0.54)^c	(7.40±0.96)^c	(6.71±0.43)^d	(7.27±0.49)^{cd}	(3.53±1.92)^b	(6.91±0.58)^{bc}	(9.22±0.27)^c
Ch120	(7.08±0.73)^b	(7.98±0.84)^c	(7.07±0.39)^c	(7.93±0.19)^c	(6.06±0.82)^d	(7.77±0.32)^d	(0.00±0.00)^a	(6.09±0.54)^{ab}	(9.35±0.20)^c

Results presented as log of gene copy number per g of cheese or mL of milk±standard deviation and log CFU per g of cheese or mL of milk±standard deviation among four farms (F1-F4) and during the ripening of Istrian cheese (M0, Ch0, Ch30, Ch60, Ch90 and Ch120, where M=milk, Ch=cheese, and the number denotes time).
The effect of farm or time on the number of investigated bacterial groups is indicated by significance level. Results of the Friedman test are presented in italic.
*significant at p<0.05, **significant at p=0.001, ns=not significant; a,b,c,d=significantly different groups (ANOVA, post hoc test); *a,b,c*=significantly different groups related to CFU count (Friedman test); *A,B*=significantly different groups related to real-time PCR count (Friedman test)

vestigated LAB were significantly lower in raw milk (p<0.05), but they remained quite unchanged over time (30–120 days, p<0.05), reaching their highest values at the final stages of cheese ripening. The number of lactococci ranged from (5.77±0.40) to (7.08±0.73) log CFU per mL or g and the highest level occurred after 120 days of ripening. Enterococci were found at the level of (4.22±0.97) log CFU per mL of raw milk, and their counts gradually increased within the first 120 days, reaching (7.07±0.39) log CFU per g of mature cheese. Lactobacilli were present in raw milk at concentrations of (2.51±1.54) log CFU per mL, and were detected at highest number after 90 days in cheese at the level of (6.71±0.43) log CFU per g. Enterobacteria were present in raw milk at the level of (2.71±1.55) log CFU per mL. Their counts peaked in 30 days and after 120 days they were below the detection level. The microbiological counts of respective bacterial groups and the corresponding Friedman test analysis (Table 2) revealed that the bacterial communities of Istrian cheese were dominated by lactoccocci and enterococci (p<0.001). The number of lactobacilli and enterobacteria was significantly lower (p<0.001), with the enterobacteria present at the significantly lower viable concentration (Table 2). The effect of ripening time has a significant influence on the dynamics of all investigated bacterial groups (p<0.05) and no statistically significant differences in the bacterial number were noticed among the investigated farms (Table 2).

Evaluation of quality and quantity of DNA extracted from dairy samples

The quality and quantity of the DNA extracted from milk and cheese samples were evaluated using a spectrophotometer and agarose gel electrophoresis. The yields proved to be homogeneous both in terms of quantity (in the range of 20–30 ng/mL) and quality (260/280 nm absorbance ratio in the range of 1.7–2.0), and any visual DNA fragmentation was noticed on 2 % agarose gel. The presence of fat in cheese did not interfere with the purification of DNA since the fat was eliminated after the first centrifugation step prior to DNA extraction.

Evaluation of primer specificity

When primer specificity was checked by Genomatix, 1000 matches could be detected for *Lactobacillus* primers, 37 for the primers used for enterococci, 9069 for *Enterobacteriaceae* and 914 matches for the detection of lactococci (data not shown). None of the pairs of primers used in this study showed 100 % specificity. For example, the primer pair used for amplification of lactococci also detected 2 sequences related to *Streptococcus agalactiae*, the primer pair used for enterococci matched also one sequence related to *Corynebacterium* and primers used for enterobacteria amplified *in silico* several sequences related to different marine or soil cellulolytic bacteria, *e.g. Shewanella*, *Alteromonas*, *Methylophaga* and *Cellvibrio*. How-

ever, the presence of all these bacterial genera has not so far been described in cheese samples, thus the risk of generating false positive results is low. The primer pair for lactobacilli showed the least specificity, amplifying sequences related to Leuconostoc spp. and Pediococcus mesenteroides. The presence of a single PCR product of the expected size for each set of primers was confirmed by quantitative PCR using a melting curve analysis and agarose gel electrophoresis, which resulted in a single product-specific melting curve and single band of expected size on agarose gels (data not shown).

Microbial detection and quantification by real-time PCR

SYBR Green or TaqMan qPCR assays were used to identify and monitor selected bacterial groups during the Istrian cheese ripening process. The correlation coefficient of the standard curves was constantly between 0.98 and 0.99 among all assays, while the slope was between –3.3 and –3.6.

All lactic acid bacteria and total bacterial biomass quantified by qPCR assays were present at low levels in milk ($p<0.05$), but increased markedly during manufacturing, reaching the highest concentration after 90 days of ripening. The counts for lactococci were between (5.60 ± 0.72) and (8.47 ± 0.29) log of gene copy number per mL or g and the highest level was detected after 90 days of ripening. The counts for lactococci were between (5.60 ± 0.72) and (8.47 ± 0.29) log of gene copy number per mL of raw milk, and their counts gradually increased within the first 120 days reaching (7.93 ± 0.19) log of gene copy number per g of mature cheese. Lactobacilli were present in raw milk at the concentration of (3.99 ± 1.00) log of gene copy number per mL, and were detected at highest number after 120 days at the level of (7.77 ± 0.32) log of gene copy number per g. Surprisingly, for enterobacteria also, a significant increase of gene copy numbers was measured, which peaked at day 30 ((8.05 ± 0.29) log of gene copy per g). At the final stage of ripening (120 days after the ripening process started), the gene copy number dropped about 2 orders of magnitude to (6.09 ± 0.54) log of gene copy number per g. Data are summarized in Table 2.

Gene copy numbers for total bacteria estimated by qPCR ranged between (7.76 ± 0.08) and (9.41 ± 0.66) log of gene copy number per mL of milk or g of cheese, indicating that on average 11 % of total 16S rRNA gene counts were related to lactococci (Table 2). The effect of ripening time had a significant influence on the dynamics of all investigated bacterial groups ($p<0.05$). Significant differences ($p<0.05$) among the investigated farms were noticed for the total number of bacteria and the number of enterococci or enterobacteria, whereas no significant differences in the numbers of lactobacilli or lactococci among different farms were recorded (Table 2). Friedman test revealed that lactococci were the predominant population in Istrian cheese ($p<0.001$), whereas there were no significant differences among the qPCR counts related to enterococci, lactobacilli or enterobacteria (Table 2).

Correlation analysis

Comparing CFU counts, which reflect viable bacteria, to gene copy numbers of dominant bacteria in Istrian cheese, satisfying levels of agreement were revealed for enterococci ($R^2=0.866$, $p<0.001$) and lactobacilli ($R^2=0.891$, $p<0.001$), and lower correlation efficiencies were noticed when lactococci ($R^2=0.609$, $p=0.001$) or enterobacteria ($R^2=0.590$, $p=0.002$) were investigated (Fig. 1).

Discussion

In the present study, cultivation-independent quantitative approaches based on the quantitative assessment of 16S rRNA gene PCR amplicons were used to estimate the abundance of LAB and enterobacteria during the ripening of Istrian cheese. The primers and probes used in this study were applied for quantification of broader range by targeting the genus rather than an individual species to assess bacterial complexity during cheese making process. Based on the measured 16S rRNA gene copy numbers, the LAB were the dominant bacterial group during the Istrian cheese production. Their number increased towards the end of the 120-day period of Istrian cheese ripening. In the LAB group, gene copy number for lactococci was the highest. Investigations of European artisanal dairy products indicated lactococci as the most commonly found LAB genus, accounting for 38 % of the bacterial isolates identified (21). According to Medina et al. (4), lactococci represent only 14 % of LAB in raw ewe's milk but they reach a high level (>10^8 CFU/g) as early as the first day of manufacturing and maintain it throughout the ripening period of many raw milk cheeses such as Italian Pecorino Siciliano cheese, Egyptian Domiati cheese or Karakačanski skakutanac produced in Croatia (1,22,23).

The gene copy numbers of enterococci were comparable with those of lactococci and with the CFU data obtained from other Mediterranean cheeses made from raw ewe's milk (24,25). The presence of high 16S rRNA gene copy numbers of enterococci in cheeses made from raw ewe's milk is probably induced by the resistance of enterococci to high concentrations of salts or acids (26). The dairy enterococci are currently under study for their proteolytic, lipolytic and aromatic characteristics, but also for the risks derived from the presence of strains able to produce biogenic amines or carrying genes encoding for virulence or antibiotic resistance. It has also been demonstrated that the inoculation with Enterococcus faecalis increased the concentrations of 3-methyl-1--butanal, diacetyl and acetoin in ewe's cheeses, thus enhancing their flavour quality and intensity (27).

The 16S rRNA gene copy numbers of lactobacilli were one log units lower than those of lactococci and enterococci counts but high enough to influence the ripening process. The number of lactobacilli progressively increased during Istrian cheese ripening. As natural microflora of the dairy products, they are initially present in low numbers (10^2 to 10^3 CFU/g) but their number increases in cheese varieties that require long ripening periods to up to 10^7 to 10^8 bacteria per g within about 3 months (28,29). Wild nonstarter lactobacilli (NSLAB)
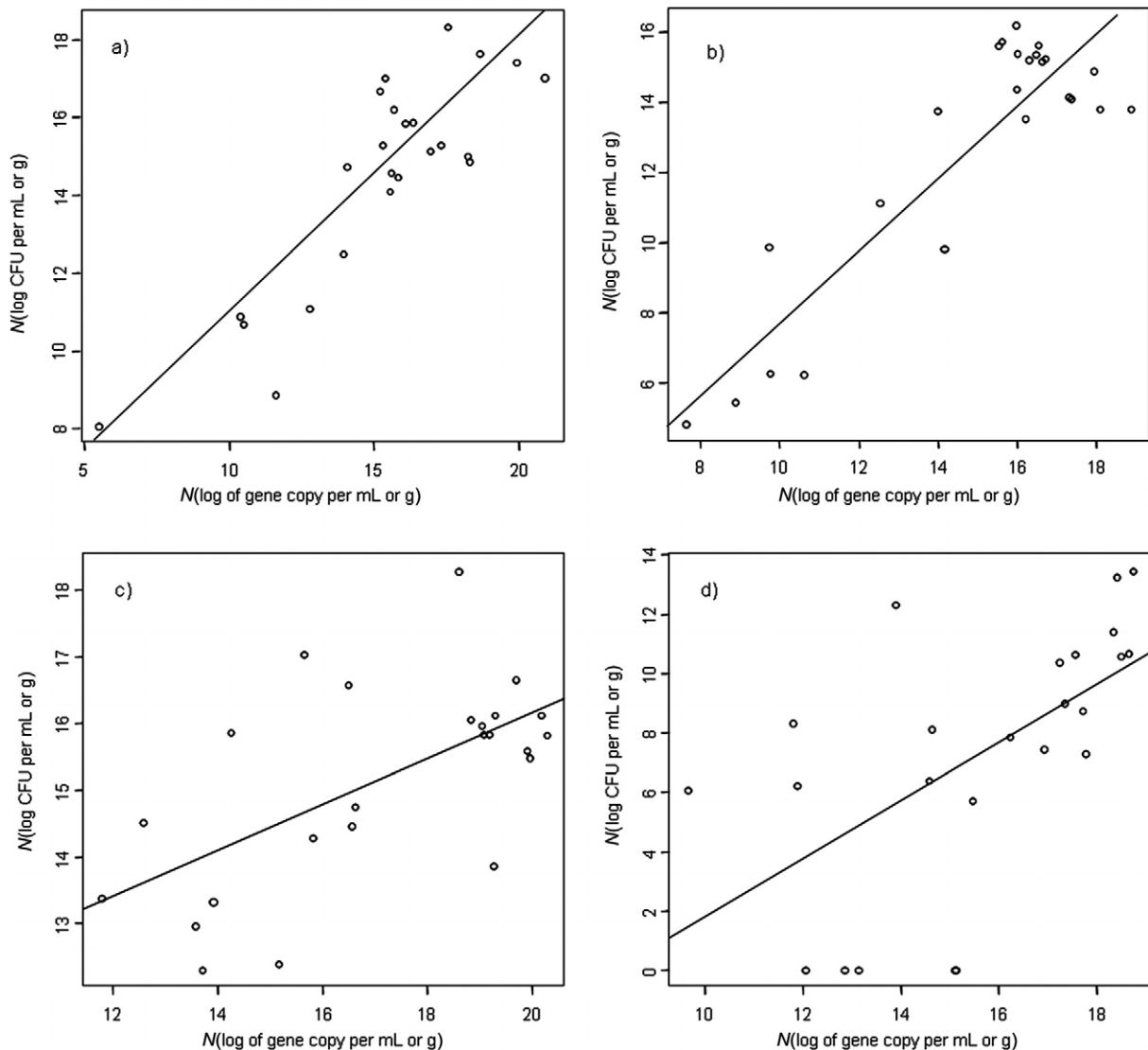
Fig. 1. Correlation between the log of gene copy number and the log CFU number of different bacterial groups: a) enterococci, b) lactobacilli, c) lactococci, and d) enterobacteria

show much lower sensitivity to salt and low water activity in comparison with the industrial starter cultures (*28*) because they are better adapted to cheese ripening conditions.

The enterobacteria had an irregular evolution when comparing CFU count and real-time PCR data. Enterobacteria counts were much lower compared with the LAB ones. At the end of ripening, CFUs of enterobacteria were below the detection limit, which may be related to the progressive increase of the lactic microbiota, to the consequent drop in pH of the cheese and to the increase in dry matter (*8*). However, the number of 16S rRNA gene copy numbers remained high. Consequently, in contrast to LAB, correlation matrices between CFU and 16S rRNA gene copy numbers were low. These results confirm the findings of earlier studies performed by several groups (*15,22*) indicating that the used media for the cultivation of microorganisms mainly select for a particular narrow group of species and that broader spectrum of species is reached by qPCR or that, simply, detection of the corresponding sequences in our study

could be attributed to dead cells, as postulated by El--Baradei *et al.* (*22*).

This example illustrates nicely that although molecular approaches have been considered as very useful and more reliable compared with classical cultivation--based methods to quantify bacteria in different environments, several concerns have been raised recently questioning this belief. They include poor DNA/RNA recovery from the complex, protein and fat-rich food matrix (*17, 30*), insufficient primer specificity as well as the inability of PCR-based methods to distinguish between the DNA from living and dead cells. Moreover, intraspecies variability and the presence of multiple copies of particular gene per genome make qPCR data interpretation more difficult, especially when the number of viable cells has to be compared with gene copy number (*31*). Considering typical problems related to the cultivation-based methods such as non-selectivity of the culture medium (*32*), the lack of growth of many bacteria in a given selective medium (*13*) or synthrophic interactions with other microbes (*30,33*), we suggest that a combined tool-

box, based on molecular approach and plate count analysis, may overcome the individual biases and generate more reliable data on the abundance of important bacterial groups involved in cheese ripening. The application of real-time PCR approach for LAB count could be especially useful when overgrowth of fast growing population (e.g. Enterococcus spp.) on a selective medium cannot be excluded, i.e. an additional method is needed to follow the in situ dynamics during the microbial succession in food matrix.

Conclusions

This study reports the use of a culture-independent approach to quantify and monitor the dynamics of bacterial populations throughout the ripening of Istrian cheese. Compared to conventional methods, culture-independent molecular methods are less time consuming, more sensitive and more specific, but except for denaturing gradient gel electrophoresis and clone library construction, they are not often used to study microbial dynamics in artisanal cheese. Real-time PCR assay can be used to trace the dominant bacterial populations during the fermentation of artisanal cheese and combined with culture-based methods, it can give more comprehensive insight into the dynamics of bacterial community throughout the cheese ripening.

References

1. C.L. Randazzo, E.E. Vaughan, C. Caggia, Artisanal and experimental Pecorino Siciliano cheese: Microbial dynamics during manufacture assessed by culturing and PCR-DGGE analyses, *Int. J. Food Microbiol. 109* (2006) 1–8.

2. J.L. Steele: Genetics and Metabolism of Starter Cultures. In: *Applied Dairy Microbiology*, E.H. Marth, J.L. Steele (Eds.), Marcel Dekker, Inc., New York, NY, USA (1998) pp. 173–193.

3. N. ben Omar, F. Ampe, Microbial community dynamics during production of the Mexican fermented maize dough pozol, *Appl. Environ. Microbiol. 66* (2000) 3664–3673.

4. R. Medina, M. Katz, S. Gonzalez, G. Oliver, Characterization of the lactic acid bacteria in ewe's milk and cheese from northwest Argentina, *J. Food Prot. 64* (2001) 559–563.

5. I. Verdier-Metz, V. Michel, C. Delbes, M.C. Montel, Do milking practices influence the bacterial diversity of raw milk, *Food Microbiol. 26* (2009) 305–310.

6. S.J. Walker, Major spoilage micro-organisms in milk and dairy products, *Int. J. Dairy Technol. 41* (1988) 91–92.

7. P. Desmerchaller, F. Fegan: *Escherichia coli*. In: *Encyclopedia of Dairy Science*, H. Roginski, W.J. Fuquay, F.P. Fox (Eds.), Academic Press, Inc., London, UK (2002) pp. 948–954.

8. C. Freitas, F.X. Malcata, Our industry today. Microbiology and biochemistry of cheeses with appélation d'origine protegée and manufactured in the Iberian peninsula from ovine and caprine milks, *J. Dairy Sci. 83* (2000) 584–602.

9. M. Zago, B. Bonvini, A.M. Martín-Platero, G. Mucchetti, D. Carminati, G. Giraffa, Characterisation of *Escherichia coli* isolated from raw milk cheeses, *Annu. Microbiol. 57* (2007) 49–54.

10. V. Lafarge, J.C. Ogier, V. Girard, V. Maladen, J.Y. Leveau, G. Delacroix-Buchet, Raw cow milk bacterial population shifts attributable to refrigeration, *Appl. Environ. Microbiol. 70* (2004) 5644–5650.

11. M. Mrkonjić Fuka, M. Engel, A. Skelin, S. Redžepović, M. Schloter, Bacterial communities associated with the production of artisanal Istrian cheese, *Int. J. Food Microbiol. 142* (2010) 19–24.

12. T.D. Leser, J.Z. Amenuvor, T.K. Jensen, R.H. Lindecrona, M. Boye, K. Møller, Culture-independent analysis of gut bacteria: The pig gastrointestinal tract microbiota revisited, *Appl. Environ. Microbiol. 68* (2002) 673–690.

13. X.W. Huijsdens, R.K. Linskens, M. Mak, S.G. Meuwissen, M. Vandenbroucke-Grauls, P.H. Savelkoul, Quantification of bacteria adherent to gastrointestinal mucosa by real-time PCR, *J. Clin. Microbiol. 40* (2002) 4423–4427.

14. R. Byun, M.A. Nadkarni, K.L. Chour, F.E. Martin, N.A. Jacques, N. Hunter, Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries, *J. Clin. Microbiol. 42* (2004) 3128–3136.

15. M. Castillo, S.M. Martín-Orúe, E.G. Manzanilla, I. Badiola, M. Martín, J. Gasa, Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR, *Vet. Microbiol. 114* (2006) 165–170.

16. J.W. He, S. Jiang, Quantification of enterococci and human adenoviruses in environmental samples by real-time PCR, *Appl. Environ. Microbiol. 71* (2005) 2250–2255.

17. C. Monnet, V. Ulve, A.S. Sarthou, F. Irlinger, Extraction of RNA from cheese without prior separation of microbial cells, *Appl. Environ. Microbiol. 74* (2008) 5724–5730.

18. H.J. Bach, J. Tomanova, M. Schloter, J.C. Munch, Enumeration of total bacteria and bacteria with genes for proteolytic activity in pure cultures and in environmental samples by quantitative PCR mediated amplification, *J. Microbiol. Methods, 49* (2002) 235–245.

19. J.A. Klappenbach, J.M. Dunbar, T.M. Schmidt, rRNA operon copy number reflects ecological strategies of bacteria, *Appl. Environ. Microbiol. 66* (2000) 1328–1333.

20. R Development Core Team: *R: A Language and Environment for Statistical Computing*, R Foundation for Statistical Computing, Austria (2008) (http://www.R-project.org).

21. T.M. Cogan, M. Barbosa, E. Beuvier, B. Bianchi-Salvadori, P.S. Cocconcelli, I. Fernandes et al., Characterization of the lactic acid bacteria in artisanal dairy products, *J. Dairy Res. 64* (1997) 409–421.

22. G. El-Baradei, A. Delacroix-Buchet, J.C. Ogier, Bacterial biodiversity of traditional Zabady fermented milk, *Int. J. Food Microbiol. 121* (2008) 295–301.

23. T. Pogačić, M. D'Andrea, D.M. Kagkli, V. Corich, A. Giacomini, E. Baldan et al., Biodiversity of microbial consortia isolated from traditional fresh sheep cheese Karakačanski skakutanac, *Mljekarstvo, 61* (2011) 208–211.

24. D.K. Papageorgiou, A. Abrahim, M. Bori, S. Doundounakis, Chemical and bacteriological characteristics of Pichtogalo Chanion cheese and mesophilic starter cultures for its production, *J. Food Protect. 61* (1998) 688–692.

25. K. Prodromou, P. Thasitou, E. Haritonidou, N. Tzanetakis, E. Litopoulou-Tzanetaki, Microbiology of »Orinotyri«, a ewe's milk cheese from the Greek mountains, *Food Microbiol. 18* (2001) 319–328.

26. J.O. Mundt, The ecology of streptococci, *Microbial Ecol. 8* (1982) 355–369.

27. B.A. Oumer, P. Gaya, E. Fernandez-Garcia, R. Marciaca, S. Garde, M. Medina, M. Nunez, Proteolysis and formation

of volatile compounds in cheese manufactured with a bacteriocin-producing adjunct culture, *J. Dairy Res. 68* (2001) 117–129.

28. V. Coeuret, S. Dubernet, M. Bernardeau, M. Gueguen, J.P. Vernoux, Isolation, characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products, *Lait, 83* (2003) 269–306.

29. A. Skelin, M. Mrkonjić Fuka, A. Čanžek Majhenič, S. Redžepović, D. Samaržija, B. Bogovič Matijašić, Phenotypic and genotypic characterization of indigenous *Lactobacillus* from traditional Istrian Ewe's Cheese, *Food Technol. Biotechnol. 50* (2012) 362–370.

30. L. Quigley, O.O'Sullivan, T.P. Beresford, R.P. Ross, G.F. Fitzgerald, P.D. Cotter, Molecular approaches to analysing the microbial composition of raw milk and raw milk cheese, *Int. J. Food Microbiol. 150* (2011) 81–94.

31. M. Mrkonjić Fuka, M. Engel, A. Hagn, J.C. Munch, M. Sommer, M. Schloter, Changes of diversity pattern of proteolytic bacteria over time and space in an agricultural soil, *Microbial Ecol. 57* (2010) 391–401.

32. M.L. Giannino, M. Marzotto, F. Dellaglio, M. Feligini, Study of microbial diversity in raw milk and fresh curd used for Fontina cheese production by culture-independent methods, *Int. J. Food Microbiol. 130* (2009) 188–195.

33. R.F. Vogel, R. Knorr, M.R. Muller, Non-dairy lactic fermentations: The cereal world, *Antonie van Leeuwenhoek, 76* (1999) 403–411.