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Evolution of the Raw Cow Milk Microflora, Especially Lactococci, Enterococci, Leuconostocs and Lactobacilli over a Successive 12 Day Milking Regime

Dalmasso Marion, Prestoz Sylvie, Rigobello Véronique and Demarigny Yann
Laboratoire de Microbiologie Alimentaire, ISARA-Lyon, Agrapole, 23,
rue Jean Baldassini, 69364 Lyon Cedex 07, France

Abstract: This research was developed to track the microbial composition of raw milk obtained from twelve successive milking operations from the same farm with a special attention to lactococci, enterococci, lactobacilli and leuconostocs. Enterococci, lactobacilli and leuconostocs were detected at low levels. Lactococci represented the most abundant microflora. The dominance of *Lc lactis* subsp. *cremoris* from days 1 to 5 was followed by the dominance of *Lc lactis* subsp. *lactis*. Technological characterizations, Rep-PCR and whole-cell protein patterns analyses revealed the existence of two groups of lactococci. The first group designed as fundamental microflora included strongly acidifying strains. They were present day after day. The second group-transitory microflora-was observed occasionally and was less acidifying. A major influence of the biofilms of the milking equipment was postulated to explain the stability and the composition of the fundamental microflora. The implication of these observations on natural whey starters' composition and cheese particularity is discussed.

Key words: Raw milk, *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris*, technological microflora, biofilms

INTRODUCTION

Cheese making implies two main successive processes. The milk is firstly clotted and the resulting curd acidified thereafter. Acidification results from the metabolism of Lactic Acid Bacteria (LAB), which transform lactose into lactic acid. This action is essential to allow the curd to reach the proper pH value and to remove the whey. LAB can originate from four different sources depending on the process: the raw milk, the cheese plant environment, commercial starters and the whey from the previous cheese making. This last practice called back slopping is still largely used in many countries and for a great variety of processes. The whey is recovered just after molding and incubated until the next cheese making process (Gatti *et al.*, 2003). During this culture, LAB level increases dramatically as a consequence of the multiplicity of genus and species. The resulting whey starter-designed thereafter as Natural Whey Starter (NWS)-consists of complex microbial associations (Reinheimer *et al.*, 1996). The LAB population originates from three main sources, (1) the previous cheese making, (2) the microbial environment of the cheese plant and (3) the raw milk microflora (Bertoni *et al.*, 2001).

The role of LAB is therefore mainly limited to the first steps of cheesemaking. During the ripening process, starter LAB are less important than other flora such as, for instance, Non Starter Lactic Acid Bacteria (NSLAB). Nevertheless, they still contribute to the development of the typical cheese aroma

Corresponding Author: Dalmasso Marion, Laboratoire de Microbiologie Alimentaire,
ISARA-Lyon, Agrapole, 23, rue Jean Baldassini, 69364 Lyon Cedex 07, France
Tel: +33 (0)4 27 85 85 85 Fax: +33 (0)4 27 85 85 86

and texture. LAB possess many enzymes-namely endo and exopeptidases-which can directly contribute to the appearance of sapid molecules, or ultimately generate aromatic compounds precursors (Mauriello *et al.*, 2001; Herreros *et al.*, 2003).

Natural Whey Starters (NWS) are particularly interesting for these two enzymatic properties. The diversity of strains which composed these bacterial communities is assumed to contribute to the enhancement of the typical flavor of many traditional cheeses and among them many products of Controlled Denomination of Origin (CDO).

If the microbial diversity of NWS is commonly described as positive, a major problem may arise in case of any technological problem. In particular, acidification defects are more and more reported by cheese makers using NWS. Such problems may originate from diverse causes and generate a great array of consequences among which, sudden stops or lengthening of the milk acidification curves frequently described. However, it is at the present time almost impossible to identify the origin of the problem and therefore to propose an appropriate solution because of the microbial complexity of NWS.

Among the four LAB sources which participate in the NWS diversity, raw milk is often described as a major, although fluctuating, source of LAB (Bachmann *et al.*, 1996; Centeno *et al.*, 1996; Manolopoulou *et al.*, 2003; Duthoit *et al.*, 2005). The influence of the raw milk microflora on the cheese characteristics has been demonstrated in the past (Herreros *et al.*, 2007), even if most of the research was not specifically focussed on NWS. At the present time, no experiment has been published on the specific influence of raw milk LAB on the complexity of NWS ecosystems. Such work faces to two main difficulties. The raw milk LAB microflora is quantitatively rather low and its contribution difficult to assess. Moreover, the study of NWS implies a dynamic approach on several consecutive days and little is known about the evolution of LAB from day to day in raw milk of the same farm origin.

Consequently, the composition of raw cow milk samples coming from the same farm was followed over 12 consecutive days. The aim of this study was to evaluate LAB biodiversity, with a specific focus on lactococci, leuconostocs, lactobacilli and enterococci, from day to day. In a future work, we will study the influence of this LAB dynamic on the composition of natural whey starters.

MATERIALS AND METHODS

Milk Sampling and Colony Enumeration

Raw cow milk samples were collected from the same farm over twelve consecutive days in February 2006. Mesophilic aerobic microflora, leuconostocs, enterococci, yeasts and moulds, total and faecal coliforms, *Pseudomonas* were enumerated respectively on PCA, MSE, KF, GGC, VRBL and CFC agar as described by Desmaures *et al.* (1997). Lactococci were checked on Turner agar (Curk *et al.*, 1994), facultative heterofermentative lactobacilli on FH-agar (Isolini *et al.*, 1990); Micrococcaceae on Mannitol Salt Agar (Demarigny, 1997) and corynebacteria on Cheese Ripening Bacterial Medium (Denis *et al.*, 2001).

After enumeration, a maximum of four colonies were selected from Turner, MSE, KF and FH agar. After microscopic examination, cocci were purified on M17 agar (Biokar diagnostics, 60000 Beauvais, France) (30°C, 24 h) and bacilli strains were purified on MRS agar (Biokar diagnostics) (anaerobiosis, 37°C, 24 h). Each isolate was finally stored at -80°C. One hundred and thirty six strains were collected and characterized from raw milks -40, 40, 40 and 16 isolates respectively from MSE, Turner, KF and FH agar.

Strains Characterisation

After thawing, lactococci, leuconostocs and enterococci were cultivated on M17 broth (Biokar diagnostics) for 24 h at 30°C and lactobacilli on MRS broth (Biokar diagnostics) for 24 h at 30°C. Cells

were then harvested and washed twice in 0.09 g L⁻¹ NaCl (Sigma) distilled water. Optical density at 600 nm was adjusted between 0.2 and 0.3. This final suspension was used to inoculate at 2% the characterization media. All the incubations were made at 30°C.

Biochemical Characterization

Biochemical Characterization of Lactococci and Leuconostocs

Salt resistance, growth at 15 and 37°C, sugars fermentation, arginine dihydrolase capability and citrate utilization were studied as described by Demarigny (1997).

Biochemical Characterization of Enterococci

Sugar fermentation was tested on API 20A basic medium (BioMérieux, 69280 Marcy l'Etoile, France). 0.5 mL of a saccharose, glycerol or L-arabinose solution (50 g L⁻¹, Sigma) was added to 4.5 mL of medium. Growth at 45°C and pH = 9.6 was performed on M17 broth. Citrate utilization was checked on KCA agar (Nickels and Leesment, 1964).

Biochemical Characterization of Lactobacilli

Catalase test, arginine dihydrolase capability, citrate utilization, growth at 15 and 45°C, production of CO₂ and sugar fermentation for the characterization of lactobacilli were performed as described by Demarigny (1997).

Technological Characterization

Isolates were all analysed for the following technological aptitudes: acidification abilities, proteolysis, autolysis and behaviour on lithmus milk as described by Demarigny *et al.* (2006).

Whole-Cell Protein Patterns

The procedure followed was described by Demarigny *et al.* (2006) to study the diversity of lactococci from natural whey starters.

Extraction of Total DNA

From a single culture broth incubated at 30°C for 24 h, total DNA was extracted by using the Nucleospin tissue kit (Macherey-Nagel, 67722 Hoerd, France).

PCR Amplification and Gel Electrophoresis

The strains were confirmed to belong to *Enterococcus*, *Leuconostoc mesenteroides* and *Lactococcus lactis* subsp. *lactis* and *cremoris* by means of PCR-based methods. Enterococcal DNA were amplified using primers E1 and E2 (Sigma), bidding to positions 632-646 and 1353-1369, respectively and corresponding to positions in the *E. coli* 16S rRNA sequence, according to Deasy *et al.* (2000). 16S rDNA fragments of *Ln. mesenteroides* were amplified using primers Lnm1, corresponding to conserved *E. coli* 16S rRNA position 185 forward and Lnm2 (position 470 reverse) (Sigma) according to Cibik *et al.* (2000). *Lactococcus lactis* subsp. *lactis* or subsp. *cremoris* DNA amplification was performed using primers His1 and His2 (Sigma), bidding to positions 671-688 and 1587-1604, respectively and corresponding to positions in *Lc. lactis* subsp. *lactis* NCDO 2118 numbering, according to Corrolier *et al.* (1998). Inter-Repetitive Extragenic Palindromic sequences were amplified by means of two 18-mer primers in combination (Rep1R-Dt, Rep2-D) (Sigma) as described by Bouton *et al.* (2002) for REP-PCR. In all cases, amplification reactions were performed in a final volume of 25 µL containing 1x reaction PCR buffer (Sigma), 0.4 µM of each opposing primers, 1 mM MgCl₂ (Sigma), 0.2 µM of each deoxynucleoside triphosphate (Sigma), 0.5U Taq DNA Polymerase (Sigma) and 5 µL of DNA. The primer sequences and the PCR amplification conditions applied are recapitulated on Table 1. Amplification cycles were performed with a Thermal Cycler (Biorad, 92430

Table 1: Primer sequences, amplification and application of PCR reactions

| Primer sequences | Amplification conditions | Application |
|------------------------------------|--|---|
| REP1R-Dt: 5'-IIINCGNCGNCATCNGGC-3' | 5 min at 94°C, 30 cycles of: 1 min at 94°C, | Rep-PCR |
| REP2-D: 5'-NCGNCTTATCNGGCCTAC-3' | 1 min at 40°C, 6 min ramping to 72°C and 1 min at 72°C | |
| Lnm1: 5'-TGTCGCATGACACAAAGTTA-3' | 5 min at 94°C, 30 cycles of: 30 sec at 94°C, | Confirmation of the species <i>Leuconostoc</i> <i>mesenteroides</i> |
| Lnm2: 5'-ATCATTTCTATTCTAGCTG-3' | 30 sec at 58°C and 1.5 min at 72°C | |
| E1: 5'-ATCACCGGGGAGGGT-3' | 5 min at 94°C, 25 cycles of: 1 min at 94°C, | Confirmation of the genus <i>Enterococcus</i> |
| E2: 5'-ATTACTAGCGATTCCGG-3' | 1 min at 60°C, 1 min at 72°C | |
| His1: 5'-CTTCGTTATGATTTTACA-3' | 5 min at 94°C, 30 cycles of: 1 min at 94°C, | Confirmation of the subspecies <i>Lactococcus lactis</i> <i>lactis</i> and <i>cremoris</i> |
| His2: 5'-AATATCAACAATCCATG-3' | 2 min at 45°C, 2 min at 72°C, 1 final step of 5 min at 72°C | |

Marne-la-Coquette, France). PCR products of 25 µL were electrophoresed in a 10 g L⁻¹ Seakem GTG agarose gel (Sigma) in TBE (Tris - Borate - EDTA pH 8) at 100 V for 3 h. The 123-pb DNA ladder (Invitrogen, 95613 Cergy Pontoise, France) was used as a size standard. The DNA fragments were stained with ethidium bromide (Sigma), viewed under UV light (302 nm, Biorad) and photographed on a digital camera (Camedia C-5060, Olympus).

Computer Analyses of REP-PCR Profiles

The band patterns were normalized and processed according to the same procedure as whole-cell protein patterns. The similarity coefficient (80%) of the Rep-PCR technique was evaluated by studying two strains six fold.

Statistical Analyses

Correlations, Multiple Correspondence Factorial Analyses (MCFA) between isolates and technological abilities and Hierarchical Classifications (HC) were performed using the STATITCF software (5th version, 1995, Institut Techniques des Céréales et des Fourrages, Paris, France).

RESULTS

Enumeration Results

The mesophilic aerobic microflora level ranged between 3.2±0.08 and 5.2±0.08 log cfu mL⁻¹. Corynebacteria and Micrococcaceae were present systematically at constant levels, respectively 3.4±0.12 and 2.2±0.16 log cfu mL⁻¹ (Fig. 1). Yeast counts were inferior to 1.0±0.2 log cfu mL⁻¹, except on days 2, 8, 11 and 12 (2-3±0.2 log cfu mL⁻¹). Moulds were detected irregularly in milk and at levels that barely exceeded 2.0±0.66 log cfu mL⁻¹, except on days 11 and 12 (over 3.0±0.66 log cfu mL⁻¹). Coliforms and *Pseudomonas* were temporarily found at levels ranging from 1.2 to 4.4±0.4 log cfu mL⁻¹.

The evolution of the four main LAB microflora was based on the use of selective-FH agar, KF-and elective-Turner, MSE-media. The selectivity of these media being erratic, it is admitted to refer to a specific population by adding the term presumed. In some cases however, the gap between presumed and real levels is too large to be relevant. As a consequence, even the additional use of presumed does not refer to any correct tendency. In this study, this difficulty was overcome by characterizing isolates from the four LAB media. 136 isolates were picked following the number of colonies enumerated each day: 16 from FH agar, 40 from each other media. These isolates were identified by means of phenotypic and PCR-based methods. The isolates from Turner and FH media were all classified as respectively *Lactococcus* and *Lactobacillus*. Thirteen isolates out 40 from MSE were identified as *Leuconostoc*, while the 27 remaining isolates were further characterized as lactococci.

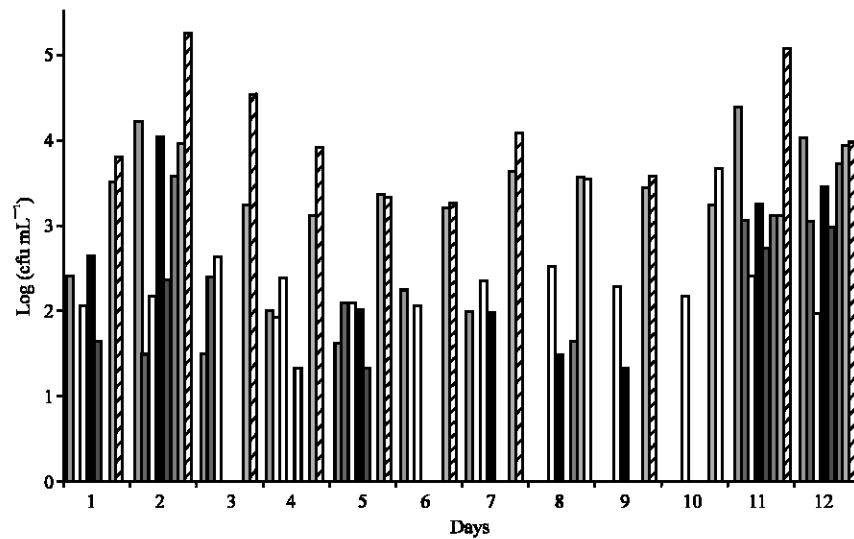


Fig. 1: Evolution of the non-LAB microflora- (■) mesophilic aerobic microflora, (■) total coliforms, (■) faecal coliforms, (■) *Pseudomonas*, (■) corynebacteria, (■) moulds, (■) yeasts, (□) Micrococaceae-in raw cow milk during twelve successive days of milking

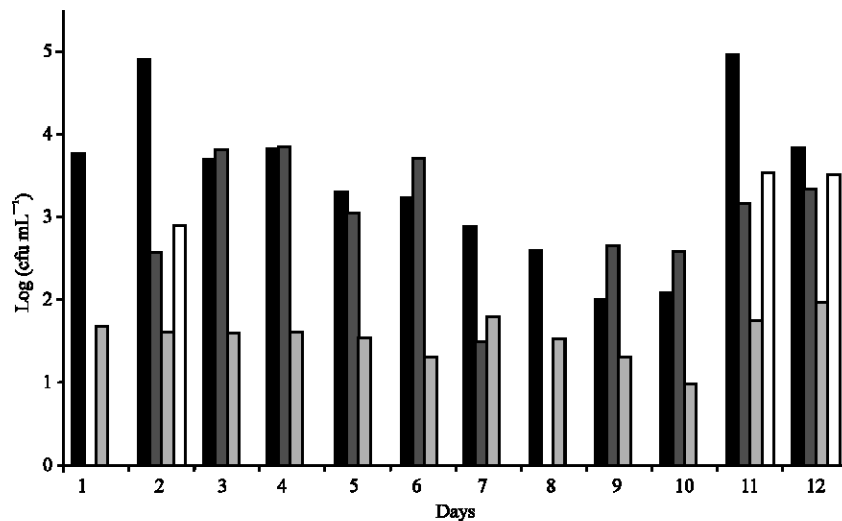


Fig. 2: Evolution of the LAB population- (■) lactococci, (■) leuconostocs, (■) enterococci, (□) lactobacilli-in raw cow milk during twelve successive days of milking

Thirty-one isolates out of 40 from KF were referred to *Enterococcus* genus. It was not possible to clearly link the nine other isolates with an identified population. These results allowed to calculate the correct levels corresponding to each population. For instance on day 1, if two isolates from KF out of four were clearly identified as enterococci, the number deduced from plate counting was weighted by a coefficient equal to 2/4 (Fig. 2).

Lactococci level was relatively stable from day 1 to day 4 (between 3.0 and $5.0 \pm 0.1 \log \text{cfu mL}^{-1}$) and decreased thereafter to reach $2.0 \pm 0.1 \log \text{cfu mL}^{-1}$ on days 9 and 10. Then, the number of lactococci increased again. Enterococci were detected every day but never overstepped $2.0 \pm 0.4 \log \text{cfu mL}^{-1}$.

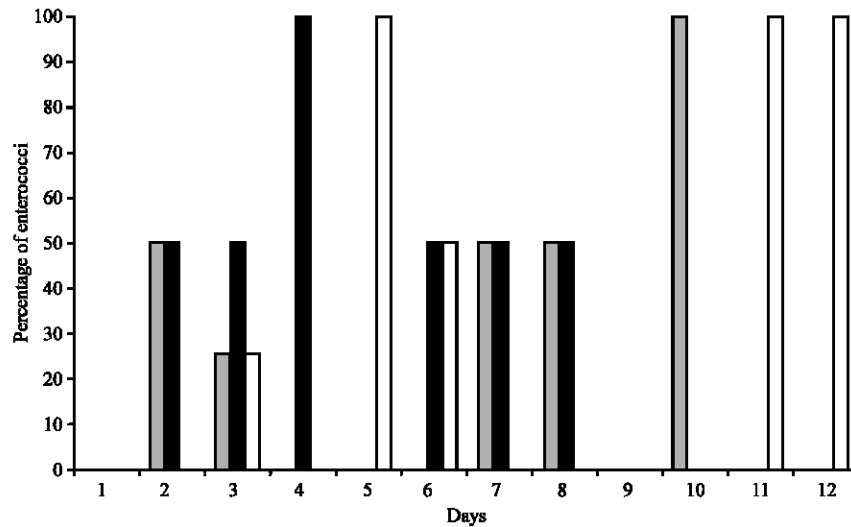


Fig. 3: Main enterococcal groups- (■) I, (■) II, (■) III-obtained by hierarchical classification for twelve days of milking

Lactobacilli were only detected on days 2, 11 and 12. Leuconostocs were detected everyday except on days 1 and 8. The levels of these two populations ranged between 2.4 and $4.0 \pm 0.3 \log \text{cfu mL}^{-1}$. A strong correlation was observed between leuconostocs and lactobacilli ($r = 0.95$).

It is noteworthy that lactococci, mesophilic aerobic microflora and total coliforms were strongly correlated ($r > 0.82$), inferring a potential common origin for these three microflora.

Isolates Characterizations

Leuconostocs and Lactobacilli

The thirteen strains belonging to the *Leuconostoc* genus appeared to have the phenotypic characteristics of *Ln mesenteroides* subsp. *mesenteroides*. Leuconostocs mean acidifying and proteolytic activities were rather weak, respectively 0.20 ± 0.02 pH units and 9.2 ± 4 $\mu\text{moles of glycine mL}^{-1}$.

Lactobacilli were characterized by phenotypic means. They all belonged to the facultative heterofermentative lactobacilli group. Further identifications indicated that the isolates were classified as *Lb plantarum* or *Lb paracasei* subsp. *paracasei*.

Enterococci

Fourteen isolates were identified as *Enterococcus faecalis*, ten as *Ec durans* and two as *Ec faecium*. Five enterococci could not be accurately identified. Distribution of these three populations during the 12 days did not reveal any specific trend. *Ec faecalis* strains were more commonly identified at the beginning of the period and *Ec durans* more common at the end. Despite this dual distribution, no correlation was observed between the three species.

Enterococci were divided into three groups-I, II, III-thanks to HC analysis (Fig. 3). The mean acidifying and proteolytic activities were equal to 0.66 ± 0.06 pH units and 3.6 ± 0.4 $\mu\text{moles of glycine mL}^{-1}$ for group I strains, 0.56 ± 0.08 pH units and 2.4 ± 0.7 $\mu\text{moles of glycine mL}^{-1}$ for group II strains and 0.26 ± 0.13 pH units and 1.7 ± 0.9 $\mu\text{moles of glycine mL}^{-1}$ for group III strains. A negative correlation was observed between III and II or III and I populations $r = -0.68$. Group III strains were generally present when the strains of the other groups were absent or at low rates.

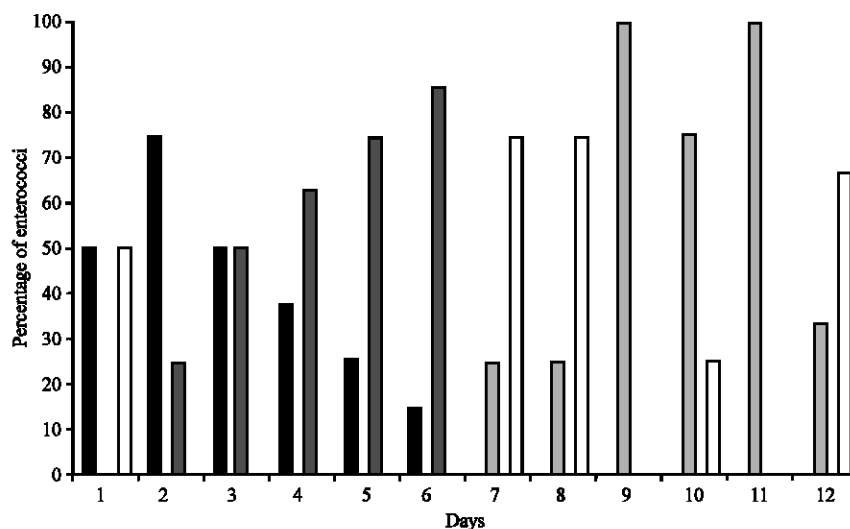


Fig. 4: Main lactococcal groups- (■) A, (▒) B, (▓) C and (□) erratic strains-obtained by hierarchical classification for twelve days of milking

Lactococci

Forty lactococci were picked from Tunes agar and 27 from MSE agar. All these isolates displayed the phenotypic features of the subspecies *Lactococcus lactis* subsp. *lactis*. For example, they all showed positive arginine dihydrolase capability. They were, also, able to grow in a 40 g L⁻¹ NaCl culture broth and to use maltose and ribose. Growth in litmus milk indicated that over 95% of the isolates were able to reduce, acidify and clot the milk in less than 24 h. None of the strains were able to use citrate to produce diacetyl.

The confirmation of the species identification was based on the identification of the histidine biosynthesis operon region, because of its *Lc lactis* species specificity. Based on its size, the resulting fragment was subspecies specific. *Lc lactis* subsp. *cremoris* strains have a DNA sequence of about 200 bp between the *orf3* and *hisC* genes, which is not present in *Lc lactis* subsp. *lactis* strains. This work showed that all the strains belonged to *Lc lactis* species. However, 50% of the strains were classified in the *cremoris* subspecies. From day 1 to day 4, only *Lc lactis* subsp. *cremoris* was present in the milk. On day 5, a change was observed, the two subspecies being equally represented. Thereafter, *Lc lactis* subsp. *cremoris* disappeared to be replaced by *Lc lactis* subsp. *lactis*.

MCFA and HC analysis allowed to separate lactococci into four main groups, A, B, C and erratic strains (Fig. 4). If autolysis was roughly identical irrespective of the strains, some slight differences were observed on the basis of proteolysis and acidification aptitudes. The mean acidifying and proteolytic activities were equal to 1.70±0.01 pH units and 6.7±0.6 µmoles of glycine mL⁻¹ for group A strains, 1.58±0.23 pH units and 5.1±1.6 µmoles of glycine mL⁻¹ for group B strains and 1.74±0.14 and 6.4±1.1 µmoles of glycine mL⁻¹ for group C strains. Strains which could not fall into one of these three groups were designed as erratic strains.

From days 1 to 6, the proportion of A strains decreased gradually in time. A strains represented 75% of the lactococci isolates on day 2 and only 15% on day 6. At the same time, group C became more and more important from day 2-25% of the isolates-to day 6-85% of the isolates. C strains suddenly disappeared thereafter, to be replaced by B strains. Erratic strains represented in some cases the dominant flora among lactococci isolates. Their presence was rather congruent with that of B strains.

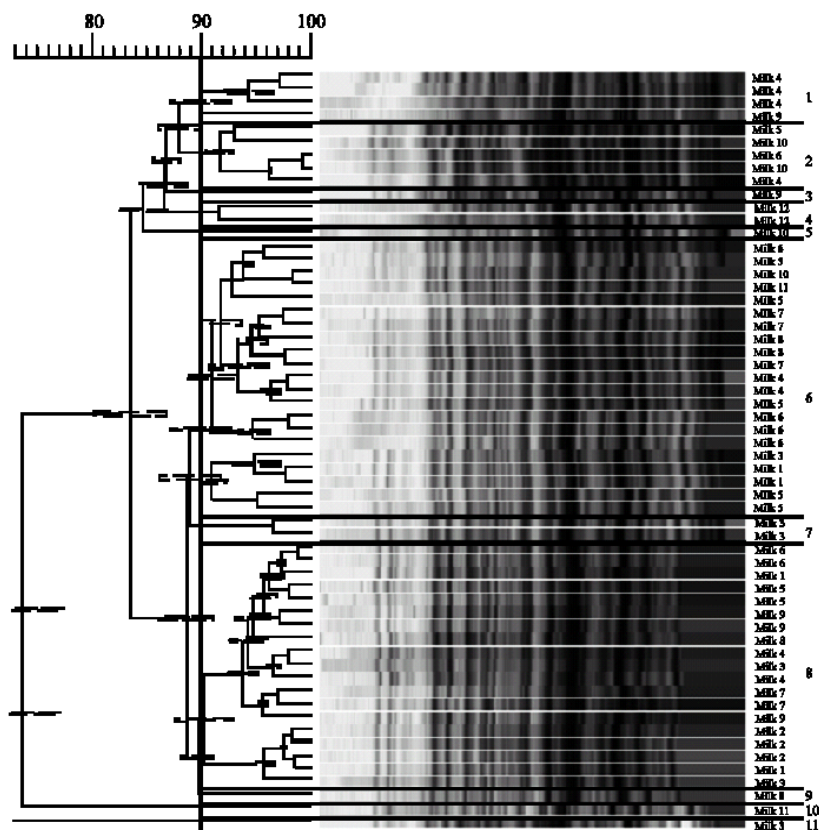


Fig. 5: Dendrogramme of 58 lactococcal whole-cell protein patterns by SDS-PAGE. Each pattern is identified by a milk number indicating the origin of the strain, e.g., all the lactococcal strains from milk 1 are labelled Milk 1 on the dendrogramme. Clusters are materialized by bold horizontal lines and numbered from 1 to 11. The similarity coefficient of 90% is materialized by a bold vertical line

The whole-cell protein patterns of the 67 lactococci were analyzed by SDS-PAGE. Nine strains providing unusable profiles were not kept. The 58 remaining patterns were divided into 11 strain clusters, on the basis of a similarity coefficient of 90% (Fig. 5). Clusters 6 and 8 grouped 21 and 19 profiles i.e., 69% of the lactococci strains. Among these two clusters, strains originated from milks 1 to 11, meaning that along this period, a fundamental microflora had settled durably. The 18 other patterns were shared among the nine remaining clusters. They corresponded with strains isolated from milks 3, 4, 6 and 8 to 12. Compared with the leading microflora represented by clusters 6 and 8, this secondary microflora could be designed as transitory.

It is interesting to note that these results partially confirmed the observations deduced from acidifying abilities and proteolysis analysis. C strains profiles were distributed among several clusters. A and B strains profiles were essentially concentrated in clusters 6 and 8. These strains could be linked with the fundamental microflora and C and erratic strains to the transitory microflora. The distribution of A, B and C strains among the clusters allowed to calculate the diversity coefficient $H = \sum P_i \log P_i$ (with P_i = number of strains in cluster i /total number of strains) (Shannon and Weaver, 1983). A, B and C populations diversity coefficients were respectively equal to 30, 54 and 71%. A population was

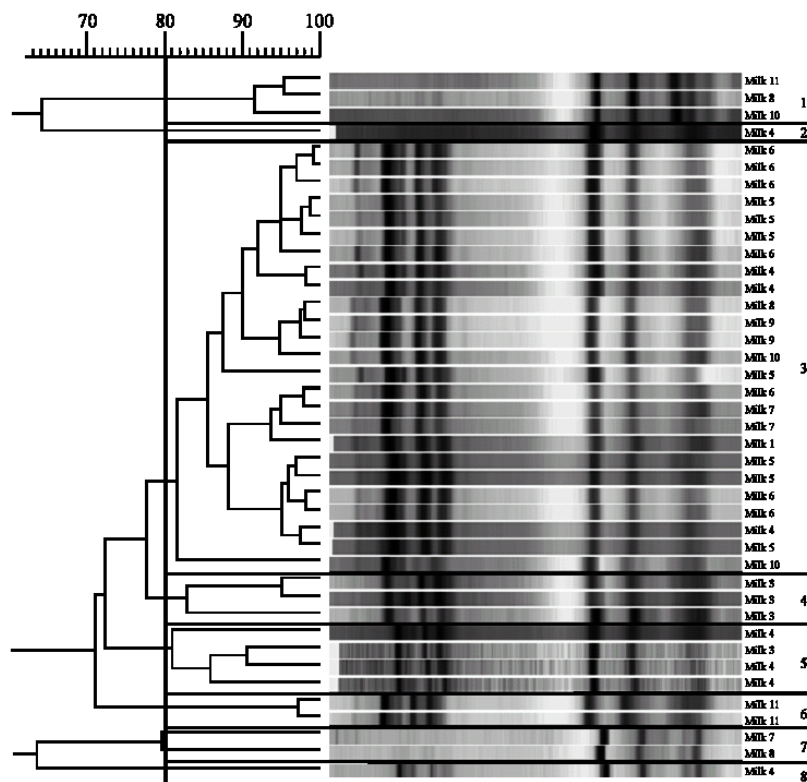


Fig. 6: Example of a dendrogramme of lactococcal profiles by REP-PCR. Each pattern is identified by a milk number indicating the origin of the strain, e.g., all the lactococcal strains from milk 1 are labelled Milk 1 on the dendrogramme. Clusters are materialized by bold horizontal lines and numbered from 1 to 8. The similarity coefficient of 80% is materialized by a bold vertical line

the less diversified, strains being shared among clusters 6 and 8. C population included six different profiles.

A second analysis was made by Rep-PCR. An example of the results obtained with this technique is displayed on Fig. 6. Eight clusters were obtained. 83% of the lactococci classified in the SDS-PAGE cluster 6 were again in the same cluster (3). This observation confirms the existence of some form of stable leading microflora in the milk from day to day. The other strains, in particular those from SDS-PAGE cluster 8, were not grouped into a specific cluster and were scattered among many clusters.

DISCUSSION

The origin of the raw milk microflora is still currently under study. The potential sources of contamination include the skin of the cow, feed dusts, milking equipment and finally the environment of the cheese plant. Among the different microflora which can be identified in raw milk, lactic acid bacteria seem particularly attractive for the cheese maker. These bacteria, namely lactococci and lactobacilli, are acknowledged to contribute to curd acidification. They are also suspected to bring interesting features-directly during the cheese making process or thereafter during the ripening stage-to the cheese. Such elements enable to give the cheese its original flavour typical of its geographical

origin. These bacteria are mainly characterized by their strong rusticity. This quality leads them to colonize the curd at the end of the cheese making process, in spite of hard selective conditions: their level is at least 10 000 times lower than the starter, some nutrients are rapidly falling in numbers-non protein nitrogen for instance-, acidification is very intense and in some cases, the technological temperature is far from their optimum.

This work was undertaken to monitor the evolution of the main LAB microflora during twelve consecutive days of milking, with a specific focus on the *Lactococcus* group. It allowed the study of the LAB microflora dynamic from day to day, which had never been done before at such an extensive scale. Three different methodologies were combined to study enterococci, leuconostoc, lactobacilli and lactococci: technological aptitudes, PCR methods and whole-cell protein patterns. Corsetti *et al.* (2003) followed a similar approach-wall-cell protein patterns, RAPD-to study sourdough lactic acid bacteria. They concluded to the effectiveness of the combination of two different methods to classify invasive microbial populations. In a recent study, we also underlined the necessity to cross two different microbial techniques to observe NWS, each method giving complementary results (Demarigny *et al.*, 2006).

On a twelve-day period, the mean mesophilic aerobic flora was centered around $4.0 \pm 0.08 \log \text{cfu mL}^{-1}$, with some variations. These values are similar to the observations already made by many authors on raw milk samples. The evidence of a correlation between lactococci, coliforms and mesophilic aerobic flora emphasizes a potential common origin. Lactococci levels, in particular, are frequently close to mesophilic aerobic flora levels. Lactococci probably originate from biofilms present in the milking equipment. This assertion is based on the results obtained by Laithier *et al.* (2004), which showed that biofilms were mainly composed of LAB, the other flora being generally-yet not systematically-far less important. In a recent study, Kagkli *et al.* (2007) established that milk enterococci and lactobacilli also originated from milking equipment. In our case, however, no correlation was observed between the levels of these two populations.

Although frequently counted, enterococci did not reach high levels in milk. Isolates were identified as *Ec faecium*, *Ec faecalis* and *Ec durans*, three species commonly found in raw milks (Aquilanti *et al.*, 2006). Their technological interest appeared limited because of low milk acidifying and proteolytic abilities, which is in accordance with Morandi *et al.* (2006). Some trends were observed following the acidifying proteolytic characteristics of the strains isolated. Lactobacilli were inconstant as observed by Bouton *et al.* (2005). These authors indicated that variations in the levels of fermentative lactobacilli could be partially explained by the type of feeding used by the dairy farmer, farm practices and general hygiene of the premises.

The specific enumeration of leuconostocs was particularly difficult to achieve. The MSE medium only allowed counting dextrane-producing strains of leuconostocs. This lack of selectivity was made worse by the pressure exerted by the other microorganisms able to grow on MSE (Corry *et al.*, 2007). In spite of this problem, we only found small numbers of *Leuconostoc mesenteroides* subsp. *mesenteroides*. This observation reinforces the assertion that bacteria from the *Leuconostoc* genus are a minor microflora in milk (Zamfir *et al.*, 2006).

Lactococci appeared to be the principal LAB population, their proportion ranging between 93 and 100% of the total LAB microflora. They, also, constituted the major part of the milk microflora. Their levels varied considerably, from 2.0 to $5.0 \pm 0.1 \log \text{cfu mL}^{-1}$. These results are congruent with former data. For instance, Corrolier *et al.* (1998) indicated that lactococci varied between 2 and $3 \log \text{cfu mL}^{-1}$, following the origin of the sample and the season.

All lactococci were confirmed to be part of the *Lactococcus lactis* species. Most of strains exhibited phenotypic characteristics of the subspecies *lactis* whereas they have been found to own the genetic profile of the subspecies *cremoris*. This observation is in complete agreement with the study completed by Ward *et al.* (1998) in which more than 70% of *Lc lactis* were found to have the

phenotype of the subspecies *lactis* and the genotype of the subspecies *cremoris*. This ambiguous taxonomic assignment of *Lc lactis* strains can be directly compared to the works of Salama *et al.* (1995). Actually, these authors reported that phenotypic changes from *lactis* to *cremoris* subspecies had been observed before. It is particularly true for the phenotypic change from arginine negative to arginine positive capability, which is attributed to transducing phage. Consequently, several methods have to be used and new phenotypic characteristics have to be investigated in order to discriminate properly the two subspecies *lactis* and *cremoris*. For example, Nomura *et al.* (1999) proposed to test the presence of γ -aminobutyric acid, a by-product only produced by the subspecies *lactis*. We can wonder, however, if the phenotypic characterisation is still a relevant method to identify lactococci.

During the 12 day period, we observed two different behaviors. From day 1 to day 6, *Lc lactis* subsp. *cremoris* was the only subspecies identified, replaced later by *Lc lactis* subsp. *lactis*. If we suppose a major influence of biofilms on the LAB population, this shift appears surprising. Indeed, dairy farm practices were identical from day to day during the twelve consecutive days. Otherwise, we can argue with Salama *et al.* (1995) that *Lc lactis* subsp. *cremoris* acquired its *lactis* characteristics inside the biofilm by transducing phages. This hypothesis would be then fundamental. It would then imply that the release of *Lactococcus* strains from biofilms would be accompanied by the concurrent release of phages. If raw milk lactococci are intended to settle and even to dominate in NWS, the origin of acidification defects during cheese making would be possibly explained by these phages from biofilms.

Moreover, in a preceding study (Demarigny *et al.*, 2004) NWS high levels of *Lc lactis* subsp. *cremoris* were observed in cheese. This population displayed the phenotypic features of the *cremoris* subspecies. Considering that *Lc lactis* subsp. *cremoris* are frequently added in commercial starter (Coppola *et al.*, 2006), it would indicate that NWS dominant populations partly originate from the added starter.

The analysis of *Lactococcus* strains patterns indicated the occurrence of a fundamental microflora, exhibiting stable and interesting technological behaviors and a transitory microflora. This observation is always congruent with the biofilm hypothesis and the observations made by Laithier *et al.* (2005). The authors reported that lactococci and more generally LAB from biofilms could be classified among ten different classes following their abilities.

Our results were confirmed by using three methodological means at the same time. Unlike Casalta *et al.* (2005), the Rep-PCR characterization partially failed to disclose different patterns. This technique may therefore be inappropriate to study the lactococci diversity from the same ecosystem. Apart from the fundamental population, the presence of SDS-PAGE patterns corresponding to particular technological features-less important acidification ability-could indicate another source of lactococci, derived from the milk bulk and not from biofilm. The diversity coefficients calculation seemed to confirm that the transitory microflora was more diverse than the fundamental microflora. This could confirm a different origin of the transitory isolates.

CONCLUSION

The dynamics and the characteristics of the main LAB populations in raw cow milk were studied on a twelve-day period of milking. It is allowed to suppose that lactococci which dominated over the other LAB exerted an inhibition effect. It will be interesting to test in another study the ability of wild raw milk lactococci to produce antimicrobial compounds, active against the other LAB microflora.

According to preceding research, we considered biofilms, which settle in the milking equipment, as the main origin of lactococci. We postulated the influence of transducing phages, as indicated by Salama *et al.* (1995) to explain the stability of the lactococci characteristics. It would imply that the

study of biofilms phages would be of a great interest to sort out cheese making defects. We can still wonder if the diversity of raw milk microflora contributing to the cheese particularity is not initially due to the exchanges of transducing phages.

Finally, it could be interesting to monitor the LAB microflora on many farms for many weeks. The LAB dynamics and specific farm practices have to be taken into account too.

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