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Culture-dependent and culture-independent methods for molecular analysis of the diversity of lactobacilli in "Camembert de Normandie" cheese

Ségolène HENRI-DUBERNET, Nathalie DESMASURES*, Micheline GUÉGUEN

Laboratoire de Microbiologie Alimentaire (EA 3213, USC INRA), Université de Caen Basse-Normandie, esplanade de la Paix, 14032 Caen Cedex, France

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Abstract – We compared a culture-dependent and a culture-independent approach for the assessment of lactobacilli community biodiversity and evolution during the production of RDO Camembert in three cheese-making factories. We used temperature gradient gel electrophoresis (TGGE) to analyse total microbial DNA and DNA from single isolates. TGGE patterns of total microbial DNA from milk and cheese showed that *Lactobacillus paracasei* subsp. *paracasei* was a dominant species in the three factories and that *Lb. plantarum* was also a dominant species in one. TGGE profiles from individual isolates confirmed that these two species were dominant, but also detected *Lb. delbrueckii* susbp. *lactis*, *Lb. acidophilus*, *Lb. delbrueckii* susbp. *bulgaricus* and *Lb. casei* subsp. *casei*. Thus, the two approaches provided complementary information.

Lactobacilli / TGGE / Camembert de Normandie cheese / culture-dependent method / culture-independent method

Résumé – Comparaison de méthodes culture-dépendante et culture-indépendante pour l'analyse moléculaire de la diversité des lactobacilles dans le Camembert de Normandie. La contribution de deux approches culture-dépendante et culture-indépendante a été étudiée dans le but d'apprécier la biodiversité et l'évolution des populations de lactobacilles au cours de la transformation de lait cru en camembert AOC dans trois fromageries. Une analyse par électrophorèse en gradient de température (TGGE) a été effectuée comparativement sur l'ADN microbien total et sur l'ADN extrait à partir d'isolats. Les profils TGGE obtenus à partir de l'ADN total de laits et de fromages ont montré que *Lactobacillus paracasei* subsp. *paracasei* était une espèce dominante au sein des trois fromageries et que *Lb. plantarum* co-dominait dans l'un des trois ateliers. Après étude des profils TGGE des isolats, ces deux espèces demeurent majoritaires mais *Lactobacillus delbrueckii* susbp. *lactis*, *Lb. acidophilus*, *Lb. delbrueckii* susbp. *bulgaricus* et *Lb. casei* subsp. *casei* sont également mis en évidence. Les deux approches ont conduit à l'obtention d'informations complémentaires.

Lactobacille / TGGE / Camembert de Normandie / méthode culture-dépendante / méthode culture-indépendante

* Corresponding author: nathalie.desmasures@ibfa.unicaen.fr

1. INTRODUCTION

The specificity of registered designation of origin (RDO) cheese is often related to the race and nutrition of dairy cows, which determine the physical and chemical properties of raw milk, and to basic traditional cheese-making practice. The dynamic microbial ecosystem is also important because RDO Camembert cheese is made with raw milk, which contains a great diversity of microorganisms: yeasts, moulds and bacteria such as lactococci, leuconostocs, corynebacteria and lactobacilli. Most of those microrganisms are able to reach 10^7 to 10^8 CFU·g^{-1} by the end of ripening. In RDO Camembert, most lactobacilli are NSLAB (non-starter lactic acid bacteria), originating from farm environments, animals, and the dairy manufacture environment. The *Lactobacillus* genus is the largest genus within the LAB group, with 92 species and 15 subspecies described to date (www.bacterio.cict.fr, 05/06/03). It has been the object of many taxonomic changes both within and between genera. These indigenous microorganisms play an important role in the organoleptic properties of cheese [6, 7, 16, 23], but little is known about the evolution and the nature of the *Lactobacillus* species present during the manufacturing and ripening of Camembert cheese. Desmasures et al. [9] counted on Rogosa medium between 10 and 10^4 CFU·mL^{-1} presumed lactobacilli in Normandy raw milks (produced in the autumn, winter and spring, and at different farms), and between 10^6 and 10^8 CFU·g^{-1} in corresponding raw milk Camembert cheeses on and after 16 d of ripening [8, 9].

Cultivation methods and phenotypic identification methods can, however, lead to a false evaluation of the lactobacilli levels and diversity. Indeed, cultivation media may not be sufficiently selective against other genera or may be too selective for some *Lactobacillus* species or strains [5]. Furthermore, phenotypic identification methods can give erroneous results depending on the reference used. New molecular methods, such as denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE), have been developed for the analysis of microbial communities without culture. These methods both involve the sequence-specific separation of amplified 16S rDNA fragments. These techniques have recently been used to evaluate the diversity of lactobacilli in cheeses [5, 6, 22, 23], sausages [4], starch fermentation [2], malt whisky fermentation [27], beer [26], faeces [24, 25, 30] and the gastrointestinal tract [29], and to identify *Lactobacillus* species [28]. Recently, a polyphasic approach showed the need for culture-independent methods to investigate the microbiology of fermented foods [1]. Miambi et al. [18] showed that direct PCR-DGGE of total microbial community DNA and culture-dependent techniques gave different results concerning the microbial assemblages in fermented cassava dough. This confirms the need to combine culture-dependent and culture-independent methods when describing microbial communities. We recently developed a species database that can differentiate between most of the 25 *Lactobacillus* species tested according to their TGGE profiles [10].

The aim of this work was to compare the contributions of culture-dependent and culture-independent approaches, combining TGGE analysis of total DNA and DNA from single isolates, to the assessment of the biodiversity and evolution of lactobacilli communities during the manufacture of RDO Camembert.

2. MATERIALS AND METHODS

2.1. Milk and cheese sampling, growth conditions and isolation of microorganisms

Samples of milk, curd or Camembert cheese were collected from three cheese factories (A, B and C) in Normandy at five stages (raw ripened milk: 0-day; curd: 1-day;

14-day; 30-day and 60-day ripened cheese (two cheeses per analysis)). In parallel, an enrichment of 20 mL of raw ripened milk (E-day) was incubated in 20 mL of acidified MRS broth (Merck, Darmstadt, Germany; acidified MRS adjusted to pH 5.4 with HCl) for 16 h at 30 °C and then plated out on AMRSA (acidified MRS agar) and LAMVAB (*Lactobacillus* anaerobic MRS agar with vancomycin and bromocresol green [13]).

For total DNA analysis, microorganisms were extracted from the food matrix as follows: 20 ± 0.1 g of cheese were placed into a sterile blender and homogenised in 180 mL of 2% tri-sodium acetate (pH 7 (Sigma Aldrich, St-Quentin-Fallavier, France)) at room temperature for 1 min at high speed. Ten-fold dilutions were immediately made in peptone salt (mixture containing 1 $g \cdot L^{-1}$ pancreatic casein peptone and 8.5 $g \cdot L^{-1}$ sodium chloride in distilled water, pH 7.0). One millilitre of each dilution was directly mixed with molten PCA milk agar (210 mL Plate Count Agar, 25 mL of 10% milk, 200 µL of 2.5% pimaricin (Sigma Aldrich)), and then incubated in aerobic conditions at 30 °C for 72 h. Two plates were inoculated with 0.1 mL of each food preparation.

For presumed lactobacilli counts, 0.1 mL of each food preparation was plated out on two different media: AMRSA and LAMVAB. Plates were incubated in anaerobic conditions at 37 °C for 48 to 72 h.

For each batch, 10 isolates of presumed lactobacilli (the first five on LAMVAB and the five following ones on AMRSA) were randomly selected from one plate at each stage studied and from enriched raw ripened milk, giving rise to a total of 60 isolates per batch.

2.2. Reference strains

Lactobacillus delbrueckii subsp. *bulgaricus* CNRZ 225, *Lb. paracasei* subsp. *paracasei* CNRZ 763, *Lb. plantarum* CNRZ 211 and *Lb. casei* subsp. *casei* CIP 102993 were purchased from the CNRZ collection (Centre National de Recherches Zootechniques, Jouy-en-Josas, France) and the CIP (Collection Institut Pasteur, Paris, France).

2.3. DNA Extraction

Isolates: the phenol-chloroform method [11] was used to isolate genomic DNA from overnight cultures of presumed lactobacilli grown in MRS broth.

Dairy samples: two methods were used depending on the type of sample.

For cheese samples, genomic DNA was extracted from 20 g of cheese as described by Ogier et al. [22].

An adapted version of the method described by Lucore et al. [15] and Ampe et al. [1] was used to extract DNA from milk. Milk (40 mL) was homogenised with 2.5 mL of 25% tri-sodium acetate for 5 min at room temperature and then centrifuged at 7500 × *g* (7 °C, 10 min, Eppendorf centrifuge 5810 R, Fisher Bioblock Scientific, Illkirch, France). The pellet was resuspended in 3 mL of 0.9% NaCl and 6 mL of zirconium hydroxide (Sigma; prepared according to Lucore et al. [15]), and vortexed for 10 min. After centrifugation at 500 × *g* (7 °C, 10 min), 1.5 mL of 0.9% NaCl was added and the cellular pellet was transferred into a clean 1.5 mL Eppendorf. The tube was centrifuged at 7000 × *g* (4 °C, 10 min), and 500 µL of a 20 $\mu g \cdot \mu L^{-1}$ lysozyme solution (Sigma) in TES buffer (50 $mmol \cdot L^{-1}$ Tris, 1 $mmol \cdot L^{-1}$ EDTA, 8.56% w/v saccharose) and 10 µL of mutanolysin solution (1 $U \cdot \mu L^{-1}$, Sigma) were added to the pellet. Samples were vortexed for 1 min and incubated for 1 h at 37 °C. A proteinase K solution (25 µL of a 10 $mg \cdot mL^{-1}$ solution; Sigma) was added to two 2-mL tubes containing the resulting suspension and incubated for 50 min at 50 °C and then for 10 min at 65 °C. Then 300 µL of warm (65 °C) buffer (0.2 $mol \cdot L^{-1}$ NaCl, 0.1 $mol \cdot L^{-1}$ Tris-HCl pH 8, 2% SDS) was added to each tube, and the tubes were incubated for 10 min at 65 °C.

Three hundred microlitres of 5 mol·L^{-1} NaCl were added and the tubes were gently mixed for 30 s, incubated at 4 °C for 10 min, and centrifuged at 7000 × *g* (4 °C, 10 min). Each supernatant was transferred into a fresh 2-mL tube. DNA was then extracted by the phenol-chloroform method.

2.4. PCR amplification

All primers used in this study were obtained from Invitrogen (Cergy-Pontoise, France). TGGE samples were prepared by PCR in a PTC 200 thermal cycler (MJC research, Waltham, USA). A 250-base pair (bp) rDNA sequence encompassing the 16S-23S-spacer region was amplified using primers LbLMA1-rev (5' CTC AAA ACT AAA CAA AGT TTC 3') and R16-1-GC (5' CTT GTA CAC ACC GCC CGT CA 3') [21] as previously described [11].

2.5. TGGE analysis

The PCR products obtained were subjected to TGGE analysis. TGGE was performed by using the Dcode system (BioRad, Ivry/Seine, France) and 16 cm × 16 cm × 1 mm gels. Gels were prepared with 8% acrylamide solution (acrylamide-bisacrylamide; 37.5:1; BioRad) and urea (final concentration = 7 mol·L^{-1}) and run with 1X TAE buffer diluted from 50X TAE buffer (2 mol·L^{-1} Tris base, 1 mol·L^{-1} glacial acetic acid, 50 mmol·L^{-1} EDTA). Twenty-five microlitres of PCR samples were loaded into wells. The samples were then subjected to electrophoresis for 12 h at 90 V with a temperature gradient of 56 °C to 62 °C (rate of 0.5 °C·h^{-1}). Gels were stained with ethidium bromide (0.5 µg·mL^{-1} of 1X TAE buffer) for 15 min, then rinsed for 20 min in 1X TAE buffer and photographed on a UV transillumination table.

2.6. PFGE analysis

2.6.1. Preparation of plugs

Lactobacilli were grown overnight in MRS broth at 37 °C. The OD_{650} was adjusted to 0.5 with TE (10 mmol·L^{-1} Tris, 1 mmol·L^{-1} EDTA). Cells (9 mL) were harvested by centrifugation for 10 min at 4 °C (7000 × *g*), washed once in 5 mL of TES buffer (10 mmol·L^{-1} Tris, 1 mmol·L^{-1} EDTA, 0.5 mol·L^{-1} saccharose) and suspended in 400 µL of 50 mmol·L^{-1} EDTA. These samples were then mixed with 700 µL of low melting point agarose (1% in 125 mmol·L^{-1} EDTA at 50 °C) and transferred immediately into the wells of the plug mould (this makes approximately 10 plugs). The moulds were then placed at 4 °C for 20 min.

2.6.2. Lysis of cells

The plugs were removed from the moulds and placed into flasks containing 5 mL of lysis buffer (10 mg·mL^{-1} lysozyme, 0.05% N-lauryl sarcosine). The flasks were incubated for at least 4 h in a 37 °C water bath.

2.6.3. Incubation with proteinase K

The lysis buffer was removed and the inserts were washed with 20 to 30 mL of TE for 20 min. This step was repeated twice. The TE was discarded and the inserts incubated overnight at 50 °C with 4 mL of proteinase K buffer (100 mmol·L^{-1} EDTA, 10 mmol·L^{-1} Tris pH 8, 1% SDS, 0.25 mg·mL^{-1} proteinase K). The proteinase K buffer was discarded and the inserts washed with 20 to 30 mL TE for 20 min. This step was repeated twice. We added 50 µL of PMSF (phenylmethylsulfonyl fluoride, 17.5 mg in 1 mL of propanol-2) and incubated the inserts for 30 min at room temperature. The PMSF was replaced by 50 µL of fresh PMSF and the inserts were incubated for 30 additional minutes at room temperature to stop the reaction. The TE and PMSF were discarded and the inserts washed with 20 to 30 mL of TE for 20 min. This step was repeated twice. The TE was discarded and the plugs placed in 50 mmol·L^{-1} EDTA. The plugs were stored at 4 °C until use.

2.6.4. Digestion

One plug was placed in each reaction tube (1.5 mL). We added 200 µL of 1X enzyme buffer and incubated the plugs for 1 h at 4 °C. Each plug was placed in a new tube with 200 µL of 1X enzyme buffer containing 20 units of restriction enzyme (*Not*I). They were then incubated for 4 h at 37 °C.

2.6.5. Electrophoresis

PFGE was performed with a CHEF DR III (BioRad) machine using 1% pulsed-field certified agarose in 0.5X TBE. Electrophoresis was carried out at a constant voltage of 6 $V \cdot cm^{-1}$ at 14 °C for 18 h, and with a pulse time of 2 to 25 s.

3. RESULTS

3.1. Bacterial counts

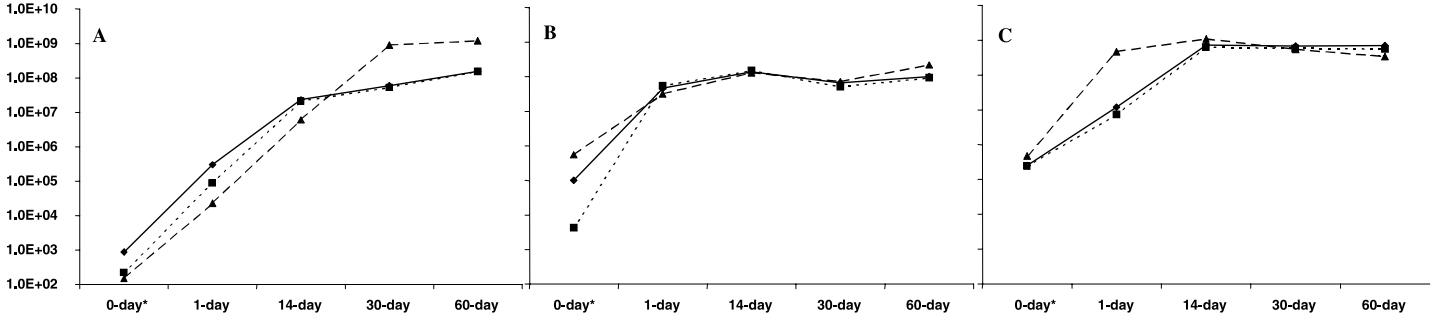
The microorganisms present in Camembert cheese during the manufacturing and ripening processes were counted on media selective for lactobacilli (Fig. 1). The initial total counts of aerobic microorganisms in raw ripened milk were 1.5×10^2, 5.5×10^5 and 4.7×10^5 $CFU \cdot mL^{-1}$ for cheese factories A, B and C, respectively. In factories B and C, aerobic microorganism counts peaked after acid development in the curd (1-day), whereas at plant A they peaked on day 30. The initial low presumptive lactobacilli counts (AMRSA or LAMVAB) increased with time, peaking on day 1 for B and day 14 for A and C. At the end of ripening, lactobacilli counts on AMRSA or LAMVAB were similar in the three factories and close to the total aerobic counts, except in plant A where the number of presumed lactobacilli was one order of magnitude lower than the total aerobic count.

3.2. PCR detection of lactobacilli during milk and cheese processing

We used PCR to amplify DNA from the entire microbial community or from single bacterial isolates for each of the three plants (Fig. 1). With the *Lactobacillus* primers (LbLMA1-rev/R16-1-GC) and total DNA samples, an amplification product was first observed on day 14 for factories A and B, and on day 1 for factory C. Total DNA from raw ripened milk gave no visible amplification products except in the case of factory C, where a 250-bp fragment was obtained after enrichment. An amplification product was obtained at all stages of manufacture when using DNA from individual bacterial isolates, with the exception of day 1 for factory B and the enriched ripened milk. The number of confirmed lactobacilli increased on day 60 for factories A and C, but was higher in the first steps of ripening (except on day 1) in factory B. The number of confirmed lactobacilli among the isolates selected on AMRSA and LAMVAB media was quite low and depended on the sampling stage and factory.

3.3. TGGE analysis of total community and single isolate DNAs

To compare the diversity and the dynamics of lactobacilli during Camembert cheese production, DNA was extracted at different stages of production (from ripened milk (including enriched ripened milk) to 60-day ripened cheese) and from the isolates obtained at every stage, and was analysed by PCR-TGGE. The TGGE patterns of total microbial DNA were similar in the three factories (Fig. 2), with one common band appearing between the fourteenth and the sixtieth day of ripening (band 3). This band comigrated with *Lactobacillus paracasei* subsp. *paracasei* CNRZ 763. The profiles of cheese from manufacturers A and B were clearly similar. The composition of the third cheese (C) was different. These patterns contained new distinct dominant amplicons (bands 1 and 2) that appeared from day 1 of manufacture and were also found in ripened milk (only after enrichment). These bands comigrated with *Lactobacillus plantarum* CNRZ 211. An additional band was detected in enriched ripened milk; it has not yet been assigned.



PCR amplification of total DNA	–	–	+	+	+	–	–	+	+	+	–	+	+	+	+
PCR amplification of isolates DNA															
LAMVAB	3	2	3	3	5	5	0	4	1	2	2	1	1	3	5
AMRSA	2	3	2	2	3	2	0	4	2	2	3	5	0	4	4
Total	5/10	5/10	5/10	5/10	8/10	7/10	0/10	8/10	3/10	4/10	5/10	6/10	1/10	7/10	9/10

* For enriched raw ripened milk, 6/10 lactobacilli isolates were obtained for the three factories.

Figure 1. Microbial counts in samples taken during Camembert cheese production in three factories (A, B and C). Bacteria were grown on PCA milk (▲), AMRSA (◆) and LAMVAB (■) agar. PCR detection of lactobacilli during milk and cheese processing with the *Lactobacillus*-primers (LbLMA1-rev/R16-1-GC) from total DNA and from DNA of bacterial isolates.

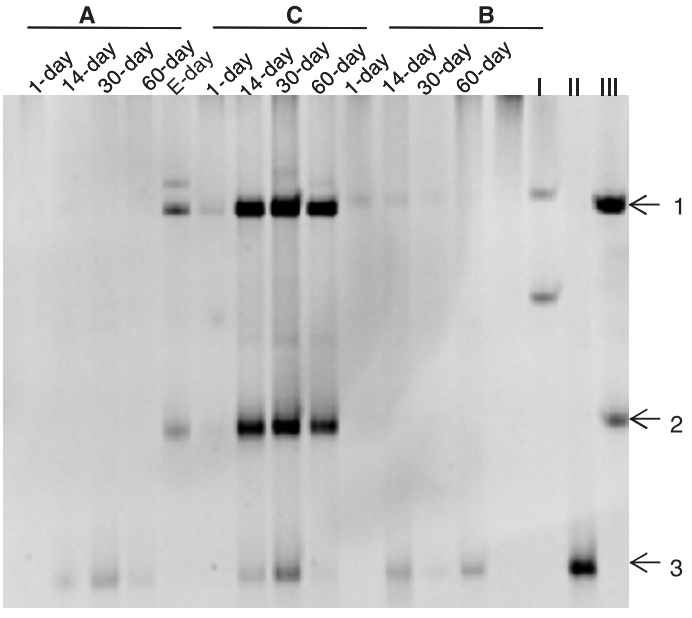


Figure 2. Identification of bacterial species present throughout the cheese-making processing in the three factories (A, B and C). TGGE was performed with the 16S-23S-spacer region rDNA fragment that was PCR amplified from extracts of enriched raw ripened milk (E-Day) and cheeses at different times of ripening (1-day, 14-day, 30-day and 60-day). Lane I: *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 225; lane II: *Lactobacillus paracasei* subsp. *paracasei* CNRZ 763; lane III: *Lactobacillus plantarum* CNRZ 211. The positions of the bands for the reference strains *Lactobacillus plantarum* CNRZ 211 (1 and 2) and *Lactobacillus paracasei* subsp. *paracasei* CNRZ 763 (3) are indicated by arrows.

TGGE analysis of 96 lactobacilli isolates revealed only six different patterns, strongly suggesting that some of the isolates shared identical sequences (data not shown). Fifty-five isolates presented the same band and comigrated with band 3 identified from TGGE analysis of total DNA in each factory. Twenty-eight isolates presented bands that corresponded to bands 1 and 2 in factory C. The other four profiles were represented by 1, 1, 5 and 4 isolates and were assigned to *Lactobacillus delbrueckii* susbp. *lactis*, *Lb. acidophilus*, *Lb. delbrueckii* susbp. *bulgaricus* and *Lb. casei* subsp. *casei*, respectively, according to our species database. Two other isolates had a pattern that could not be assigned with our species database.

3.4. PFGE analysis

We used PFGE to type 14 isolates selected at different stages and presenting the same TGGE pattern as *Lb. paracasei* subsp. *paracasei* CNRZ 763, which was found in all three factories. The PFGE profiles of the different isolates were all different from each other and from the control strains, with the exception of two isolates from factory B that shared the same pattern, UCMA 5174 and 5196 from enriched milk and 14-day ripened cheese, respectively (data not shown).

4. DISCUSSION

Our results, obtained by culture-independent and culture-dependent methods,

show and compare two different pictures of the dynamics of lactobacilli populations and species in cheese.

The PCR method using total DNA was unable to detect *Lactobacillus* populations in raw ripened milk samples, whereas that using DNA from individual isolates sometimes detected *Lactobacillus* in the same samples from all three factories. Several hypotheses may explain this discrepancy. Firstly, these methods cannot detect species representing 1% or less of the total DNA concentration [14, 20, 22]. Secondly, in our laboratory, when we inoculated milk with a mixture containing equal amounts (10^3 or 10^6 CFU·mL^{-1}) of three *Lactobacillus* species, we did not obtain a DNA amplification product with total DNA extracted from the milk inoculated with 10^3 CFU·mL^{-1} [10], which is higher than the lactobacilli levels encountered in Normandy raw milks [9]. Thus, the detection of *Lactobacillus* in milk may be limited either by low DNA concentrations or by the presence of high concentrations of competing DNA. Thirdly, the difference in the relative abundance of community members may affect the detection of certain species due to competition during PCR [14, 22]. We found that when milk was inoculated with 10^6 CFU·mL^{-1} of each of the three *Lactobacillus* species, only two of the three species could be seen on the resulting TGGE patterns. Thus, competition can occur, especially between closely-related species [10].

The culture-dependent PCR method detected lactobacilli in ripened milk (0-day) from the three factories and in 1-day cheese in factories A and C. However, the fact that lactobacilli were not detected by PCR on total DNA suggests that they were present at a low level, which is in contradiction with the enumeration results (Fig. 1), except for ripened milk (0-day) from plant A (counts $<10^3$ CFU·mL^{-1}) and 1-day cheese from factory C (lactobacilli detected from total DNA). For example, lactobacilli counts were estimated to reach levels of about 10^5 and 10^7 CFU·g^{-1} in 1-day cheese from factories A and B, respectively. This difference between the results obtained with the culture-dependent and independent approaches can be explained by two hypotheses. First, it could be due, especially for factory B, to the fact that elective or selective media, such as AMRSA and LAMVAB, were used. Few media are useful for the differential counting of lactobacilli because numerous other microorganisms including *Lactococcus, Enterococcus, Leuconostoc, Weissella, Bifidobacterium* and *Pediococcus* grow on media similar to those used for lactobacilli [5]. MRS remains the most common medium used for the isolation of lactobacilli. LAMVAB is highly selective due to its low pH and the presence of vancomycin (20 mg·L^{-1}). It is the most specific medium known for lactobacilli. Unfortunately, some lactobacilli species are vancomycin-sensitive, such as *Lactobacillus delbrueckii,* and some strains of *Lb. acidophilus* [3, 13] and leuconostocs and pediococci are also vancomycin-resistant. Although counts of presumed lactobacilli were quite high in milk, many of the colonies picked were not identified as lactobacilli. Second, it could be due to the fact that although lactobacilli counts were high (about 10^5 CFU·g^{-1} in factory A) they could not be detected among the total DNA because of the presence of "non-*Lactobacillus*" competing DNA.

We compared the biodiversity of lactobacilli species according to the culture-dependent and independent methods. *Lactobacillus delbrueckii* susbp. *bulgaricus*, *Lb. casei* subsp. *casei*, *Lb. delbrueckii* susbp. *lactis*, *Lb. acidophilus*, *Lb. plantarum* and *Lb. paracasei* subsp. *paracasei* were identified by culture-dependent PCR-TGGE whereas only *Lb. plantarum* and *Lb. paracasei* subsp. *paracasei* were detected by culture-independent PCR-TGGE. Both methods found that the same species were dominant, except in the case of enriched ripened milk where the analysis of total DNA revealed an unidentified band. Information about subdominant species was

only generated by the culture-dependent method. The total DNA method may have failed to reveal these species due to biases introduced during DNA isolation and/or PCR amplification that may have favoured certain bacteria and sequences. Alternatively, it may depend on whether identified species are dominant or subdominant. For the culture-dependent method, the picture obtained was strongly linked to the selection of isolates. Whatever the approach chosen, the use of TGGE to identify species is hampered by: the (i) comigration of bands of different sequences in TGGE analysis, meaning that other primers must be used or bands must be sequenced; (ii) the reproducibility of TGGE patterns, which requires the use of species control or an identification ladder; and (iii) taxonomic changes in the *Lactobacillus* genus (reclassification of species and introduction of new species).

Both approaches showed that *Lb. paracasei* subsp. *paracasei* was a dominant species in the three factories and that *Lb. plantarum* was also dominant in factory C. When analysing raw milk Camembert cheese by TGGE, Ogier et al. [22] detected eight bands, two of which were found to be *Lb. plantarum* and *Lb. casei*. Given that *Lb. paracasei* and *Lb. casei* are closely-related species [28], our results seem to be in agreement with this study. In 1989, we used phenotypic methods to study the diversity of LAB in RDO Camembert from factory A (ten isolates per stage of ripening) (Guéguen, personal communication). The main species identified were the same as in this study, but *Lb. brevis* was also found to be dominant in milk and curd. The fact that this species was no longer detected here may be explained by several hypotheses. First, no *Lb. brevis* isolates were selected because they grew poorly on the media used, notably on LAMVAB. Second, *Lb. brevis* was present but its DNA was not amplified from total DNA because of DNA competition. Third, due to a decrease in the diversity of the raw milk produced nowadays, *Lb. brevis* may no longer be present or dominant.

We used PFGE to investigate the diversity within the *Lb. paracasei* isolates obtained in this study. Thirteen different patterns were obtained, showing that although only a small number of species were dominant, a large number of strains were present throughout the cheese-making procedure and in the three factories.

The combination of culture-dependent and culture-independent methods helped to overcome the several limitations inherent in each of them. PCR-TGGE on total DNA can characterise microbial communities quickly and easily and can be used to study species diversity in predominant members of a complex ecosystem [12, 17–19, 22]. It can also detect the not immediately culturable (NIC) microorganisms. However, this method also has several limitations as described above, such as: (i) DNA isolation and amplification might be distorted in favour of certain bacteria and sequences; (ii) comigration of bands of different sequence or formation of multiple band patterns for one species may be particularly problematic in complex ecosystems [22]; and (iii) non-detection of the subdominant microflora (1% or less of the total community) [14, 20]. As the culture-dependent approach can detect subdominant species, it reveals a greater diversity as long as NIC microorganisms do not account for a large proportion of the microbial population. Moreover, this method is required when the aim is to characterise isolates.

In conclusion, PCR-TGGE on total DNA from milk and cheese samples is a good tool for describing the dominant *Lactobacilli* species, and the culture-dependent method can be used to describe the diversity of the lactobacilli population throughout the Camembert cheese-making procedure.

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REFERENCES

[1] Ampe F., Ben Omar N., Moizan C., Wacher C., Guyot J.P., Polyphasic study of the spatial distribution of microorganisms in Mexican pozol, a fermented maize dough, demonstrates the need for cultivation-independent methods to investigate traditional fermentations, Appl. Environ. Microbiol. 65 (1999) 5464–5473.

[2] Ampe F., Sirvent A., Zakhia N., Dynamics of the microbial community responsible for traditional sour cassava starch fermentation studied by denaturing gradient gel electrophoresis and quantitative rRNA hybridization, Int. J. Food Microbiol. 65 (2001) 45–54.

[3] Charteris W.P., Kelly P.M., Morelli L., Collins J.K., Selective detection, enumeration and identification of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in mixed bacterial populations, Int. J. Food Microbiol. 35 (1997) 1–27.

[4] Cocolin L., Manzano M., Cantoni C., Comi G., Development of a rapid method for the identification of *Lactobacillus* spp. isolated from naturally fermented Italian sausages using a polymerase chain reaction-temperature gradient gel electrophoresis, Lett. Appl. Microbiol. 30 (2000) 126–129.

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

[6] Coppola S., Blaiotta G., Ercolini D., Moschetti G., Molecular evaluation of microbial diversity occurring in different types of Mozzarella cheese, J. Appl. Microbiol. 90 (2001) 414–420.

[7] Demarigny Y., Beuvier E., Dasen A., Duboz G., Influence of raw milk microflora on the characteristics of Swiss-type cheeses. I. Evolution of microflora during ripening and characterization of facultatively heterofermentative lactobacilli, Lait 76 (1996) 371–387.

[8] Desmasures N., Étude de laits de haute qualité : Caractéristiques et aptitudes microbiologiques à la transformation en camembert au lait cru, Thèse de Doctorat, Université de Caen, France, 1995.

[9] Desmasures N., Bazin F., Guéguen M., Microbiological composition of raw milk from selected farms in the Camembert region of Normandy, J. Appl. Microbiol. 83 (1997) 53–58.

[10] Dubernet S., Maffre F., Desmasures N., Guéguen M., Identification by PCR-TGGE of species belonging to the *Lactobacillus* genus directly from dairy products, in: Congrilait 2002, 26th IDF World Dairy Congress, Paris, France 24–27 September 2002.

[11] Dubernet S., Desmasures N., Guéguen M., A PCR-based method for identification of lactobacilli at the genus level, FEMS Microbiol. Lett. 214 (2003) 271–275.

[12] Fasoli S., Marzotto M., Rizzotti L., Rossi F., Dellaglio F., Torriani S., Bacterial composition of commercial probiotic products as evaluated by PCR-DGGE analysis, Int. J. Food Microbiol. 82 (2003) 59–70.

[13] Hartemink R., Domenech V.R., Rombouts F.M., LAMVAB – A new selective medium for the isolation of lactobacilli from faeces, J. Microbiol. Meth. 29 (1997) 77–84.

[14] Heuer H., Smalla K., Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) for studying soil microbial communities, in: van Elsas J.D., Trevors J.T., Wellington E.M.H. (Eds.), Modern Soil Microbiology, Marcel Dekker, New York, USA, 1997, pp. 353–373.

[15] Lucore L.A., Cullison M.A., Jaykus L.A., Immobilization with metal hydroxides as a means to concentrate food-borne bacteria for detection by cultural and molecular methods, Appl. Environ. Microbiol. 66 (2000) 1769–1776.

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

[17] Meroth C.B., Walter J., Hertel C., Brandt M.J., Hammes W.P., Monitoring the bacterial population dynamics in sourdough fermentation processes by using PCR-denaturing gradient gel electrophoresis, Appl. Environ. Microbiol. 69 (2003) 475–482.

[18] Miambi E., Guyot J.P., Ampe F., Identification, isolation and quantification of representative bacteria from fermented cassava dough using an integrated approach of culture-dependent and culture-independent methods, Int. J. Food Microbiol. 82 (2003) 111–120.

[19] Muyzer G., Smalla K., Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology, Antonie van Leeuwenhoek 73 (1998) 127–141.

[20] Muyzer G., De Waal E.C., Uitterlinden A.G., Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA, Appl. Environ. Microbiol. 59 (1993) 695–700.

[21] Nakagawa T., Shimada M., Mukai H., Asada K., Kato I., Fujino K., Sato T., Detection of alcohol-tolerant hiochi bacteria by PCR, Appl. Environ. Microbiol. 60 (1994) 637–640.

[22] Ogier J.C., Son O., Gruss A., Tailliez P., Delacroix-Buchet A., Identification of the bacterial microflora in dairy products by temporal temperature gradient gel electrophoresis, Appl. Environ. Microbiol. 68 (2002) 3691–3701.

[23] Randazzo C.L., Torriani S., Akkermans A.D.L., De Vos W.M., Vaughan E.E., Diversity, dynamics, and activity of bacterial communities during production of an artisanal Sicilian cheese as evaluated by 16S rRNA analysis, Appl. Environ. Microbiol. 68 (2002) 1882–1892.

[24] Simpson J.M., McCracken V.J., Gaskins H.R., Mackie R.I., Denaturing gradient gel electrophoresis analysis of 16S ribosomal DNA amplicons to monitor changes in fecal bacterial populations of weaning pigs after introduction of *Lactobacillus reuteri* strain MM53, Appl. Environ. Microbiol. 66 (2000) 4705–4714.

[25] Tannock G.W., Munro K., Harmsen H.J., Welling G.W., Smart J., Gopal P.K., Analysis of the fecal microflora of human subjects consuming a probiotic product containing *Lactobacillus rhamnosus* DR20, Appl. Environ. Microbiol. 66 (2000) 2578–2588.

[26] Tsuchiya Y., Kano Y., Koshino S., Identification of lactic acid bacteria using temperature gradient gel electrophoresis for DNA fragments amplified by polymerase chain reaction, J. Am. Soc. Brew. Chem. 52 (1994) 95–99.

[27] Van Beek S., Priest F.G., Evolution of the lactic acid bacterial community during malt whisky fermentation: a polyphasic study, Appl. Environ. Microbiol. 68 (2002) 297–305.

[28] Vasquez A., Ahrne S., Pettersson B., Molin G., Temporal temperature gradient gel electrophoresis (TTGE) as a tool for identification of *Lactobacillus casei*, *Lactobacillus paracasei*, *Lactobacillus zeae* and *Lactobacillus rhamnosus*, Lett. Appl. Microbiol. 32 (2001) 215–219.

[29] Walter J., Tannock G.W., Tilsala-Timisjarvi A., Rodtong S., Loach D.M., Munro K., Alatossava T., Detection and identification of gastrointestinal *Lactobacillus* species by using denaturing gradient gel electrophoresis and species-specific PCR primers, Appl. Environ. Microbiol. 66 (2000) 297–303.

[30] Walter J., Hertel C., Tannock G.W., Lis C.M., Munro K., Hammes W.P., Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis, Appl. Environ. Microbiol. 67 (2001) 2578–2585.