Lait 80 (2000) 247–254

Original article

Evolution of *Lactococcus* strains during ripening in Brie cheese using Fourier transform infrared spectroscopy

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(Received 3 March 1999; accepted 30 July 1999)

Abstract — The diversity of the *Lactococcus* flora during maturation of soft cheese, produced using one of 2 different starter cultures (S_A and S_B), was determined using Fourier transform infrared spectroscopy (FTIR). Identification of *Lactococcus* sp. was achieved using a model composed of the 6 strains of *Lc. lactis* ssp. *lactis* and the 2 strains of *Lc. lactis* ssp. *cremoris*, present in the starter cultures S_A and S_B, as well as a reference strain for each of the subspecies considered. For the cheeses made with the starter culture S_A, the proportion of strains within the cheeses was relatively homogeneous throughout maturation, while in the cheeses made with the starter culture S_B, one strain predominated. However, with both starters the strains of *Lc. lactis* ssp. *cremoris* failed to develop in the milk culture and in the cheeses. Results demonstrated that FTIR spectroscopy is a rapid and robust method for the qualitative analysis of cheese flora.

infrared spectroscopy / *Lactococcus* flora / soft cheese / ripening

Résumé — Application de l'infrarouge à transformée de Fourier au suivi de la flore lactocoques dans le Brie. L'identification de la flore lactocoque au cours de l'affinage de fromages type Brie, résultant de l'ensemencement avec 2 levains différents, S_A et S_B, a été déterminée par spectroscopie infrarouge (IR-TF). L'identification des lactocoques composant la flore des fromages a été effectuée à partir d'un modèle discriminant composé des 6 souches de *Lactococcus lactis* ssp. *lactis* et de 2 souches de *Lc. lactis* ssp. *cremoris* présentes dans S_A et S_B et d'une souche de référence pour chacune de ces sous-espèces. Pour les fromages fabriqués avec le levain S_A, la proportion initiale des souches varie peu au cours de l'affinage, alors que pour ceux fabriqués avec le levain S_B, une des souches devient rapidement prédominante. Dans les 2 cas, la souche *Lc. lactis* ssp. *cremoris* présente dans le levain se développe peu dans le lait ensemencé et peu ou pas dans les fromages. La spectroscopie se révèle être une méthode rapide et fiable pour l'analyse qualitative de la flore des fromages en cours d'affinage.

spectroscopie infrarouge / flore lactocoque / fromage / affinage

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1. INTRODUCTION

Lactococci are used in the dairy industry as starter bacteria for their ability to grow during cheese maturation and for their contribution to the release of proteolytic enzymes by cell autolysis. The identification of microorganisms by conventional phenotypic procedures based on morphology and on biochemical tests is time consuming, involves the use of a large variety of methods and is generally unable to discriminate microorganisms at the strain level without a genotypic determination.

Fourier transform infrared spectroscopy (FTIR) has been presented as an alternative to the traditional taxonomic procedures in clinical applications [11], and to discriminate and identify pathogens [1, 6, 8] and spoilage contaminant bacteria in food [3]. In this study, the ability of FTIR to discriminate microorganisms was used to follow the evolution of *Lactococcus* strains during ripening in Brie cheese made with 2 different *Lactococcus* starter cultures.

2. MATERIALS AND METHODS

2.1. Bacterial strains

Two *Lactococcus* starter cultures, S_A and S_B, were prepared respectively with 3 different strains of *Lc. lactis* ssp. *lactis* L1, L2, L3 for S_A and L4, L5, L6 for S_B, and 1 strain of *Lc. lactis* ssp. *cremoris* C1 for S_A and C2 for S_B. Representative strains of *Lc. lactis* ssp. *lactis* CNRZ142 and *Lc. lactis* ssp. *cremoris* CNRZ105 were obtained from INRA (Station de recherches laitières de Jouy-en-Josas, France). These 10 strains are called hereafter reference strains.

2.2. Cheese making

Cheeses were manufactured in 3 experimental days according to the usual procedures for Brie cheese [10]. In each experiment, pasteurised (30 s at 76 °C) cows' milk was divided into two 10 L vats. Milk was inoculated at 37 °C with (1%) *Streptococcus salivarius* ssp. *thermophilus* starter TA 052 (Texel, Rhône–Poulenc, Dangé Saint-Romain, France) and with (1%) the *Lactococcus* starter cultures S_A or S_B. One hour after starter inoculation, the milk was coagulated with rennet. Three cheeses per vat were obtained for each day. The cheeses were salted and ripened at 12 °C for 30 d.

In order to validate the study, a control cheese was manufactured in an industrial dairy plant using the S_B culture and analysed for comparison.

2.3. Cheese analysis

2.3.1. Chemical analysis

Dry matter was measured by the FIL-IDF method [4], fat by the butyrometric method of Heiss [5], and salt content was determined with a Corning 926 chloride meter, as previously described by Bouton et al. [2]. Analyses were performed after 30 d of ripening.

2.3.2. Microbiological analysis

The experimental cheeses were analysed at 1, 15 and 30 d and the control cheese at 15, 30 and 60 d. A different cheese from the same vat was taken at each sampling step. A core sample (10 g) was dissolved in dipotassium hydrogen phosphate solution (4%) and adjusted to 100 g.

Lactococci were counted on TSE agar after 48 h incubation at 30 °C [15], in order to discriminate the *Lc. lactis* ssp. *lactis* (red colony) from the *Lc. lactis* ssp. *cremoris* (pink colony). In addition, results were confirmed by the arginine-dihydrolase test [7].

2.3.3. Sample preparation and FTIR analysis

In all cases, 25% of the colonies per plate with a maximum of 25 single colonies were

randomly harvested from TSE agar. Colonies which were assumed to belong to *Lc. lactis* ssp. *cremoris* were systematically taken (1 or 2 per agar plate). After subculture on M17 media to confirm the purity, the colonies, hereafter called isolates, were stored in M17 broth and glycerol at –20 °C.

Each colony was streaked onto M17 agar plates and grown for 48 h at 30 °C [14]. The procedure applied to prepare the sample and record a spectrum is described in Figure 1. A single colony was inoculated into M17 broth and grown for 8 h at 30 °C. The cells were then pelleted by centrifugation and washed twice with distilled water before resuspension in water to give a concentration of 40 mg of wet cells per mL. An aliquot of the fresh bacterial suspension (50 µL) was placed on a zinc selenide (ZnSe) optical plate and dried for 45 min at 50 °C. The spectra were recorded between 1 800 and 750 cm^{-1} on a Nicolet 740 spectrometer equipped with a DTGS (deuterated triglycine sulfate) detector, by co-addition and averaging of 100 scans. Spectral resolution was 2 cm^{-1} and data point resolution was 1 cm^{-1}. Two bacterial suspensions of 2 different colonies from the same agar plate were prepared for each isolate, and 3 for the reference strains. For the isolates, 1 spectrum was recorded per suspension (2 spectra), but 2 for the reference strains (6 spectra).

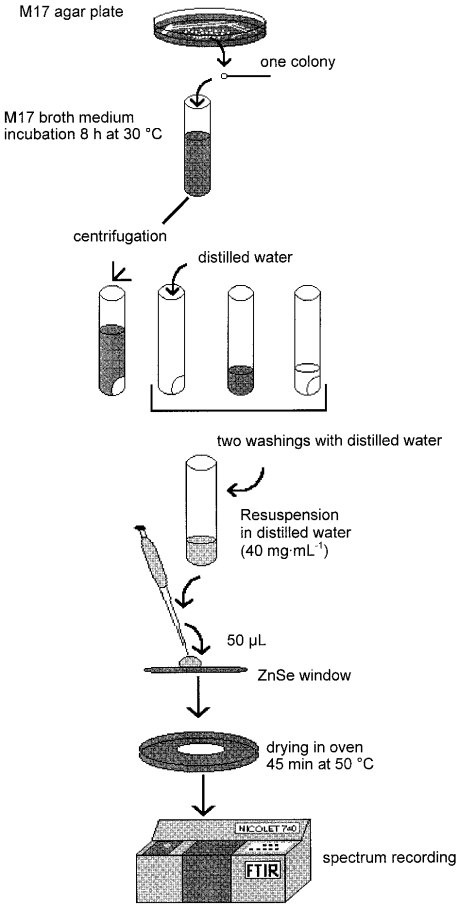

Figure 1. Sampling preparation for recording the infrared spectrum of a micro-organism.

Figure 1. Procédure de préparation d'un film bactérien en vue de l'enregistrement d'un spectre infrarouge.

2.4. Identification of isolates

The data treatment was carried out according to the discriminant analysis procedure described by Holt et al. [6] with SAS software (Statistical Analysis System, version 6.11 [12]). The discrimination between the strains was evaluated by the Mahalanobis distances method.

Prior to identification of the isolate, a model was set up using the reference strains. Discrimination was performed using 10 groups, with 1 per reference strain which are well known. Each group was composed of the 6 replicate spectra recorded. This procedure allowed fitting of the discriminant functions used afterwards, to allocate unidentified isolates to 1 of the 10 groups according to their spectral information. An isolate was considered to be correctly classified when both duplicate sampling spectra were classified in the same group. In the case of a discrepancy between duplicates, a second analysis was carried out using 2 new cell suspensions.

3. RESULTS

3.1. Spectral features of the 2 subspecies

Figure 2a gives an example of the normalised infrared spectra for the 2 CNRZ representative strains from each species. Figure 2b, with superimposed spectra shows, for 2 specific spectral areas, the slight but significant differences in spectral features between the infrared information of the 2 CNRZ reference strains.

3.2. The total *Lactococcus* flora

Figure 3 shows the variation in the total *Lactococcus* flora for each cheese throughout ripening. The 3 cheeses made with the same starter culture presented the same populations. We noticed a weak difference (0.5 log) between the 2 starter cultures. The cheeses made using the S_B culture had the lowest populations. In both cases the populations did not vary during the ripening period.

3.3. The strain discrimination model

The canonical variate analysis of spectral data shows (Fig. 4) a clear discrimination between the reference strains in the first 3 canonical axes. Table I gives the intergroup Mahalanobis distances between strains when the model was constructed from 6 spectra per strain. All the distances are higher than 24 and confirm the discrimination observed in Figure 4. Nonetheless, the discriminant model stressed the variability of the infrared spectral features of the *Lc. lactis* ssp. *lactis* group, with 2 strains, L1 and L6, which have Mahalanobis

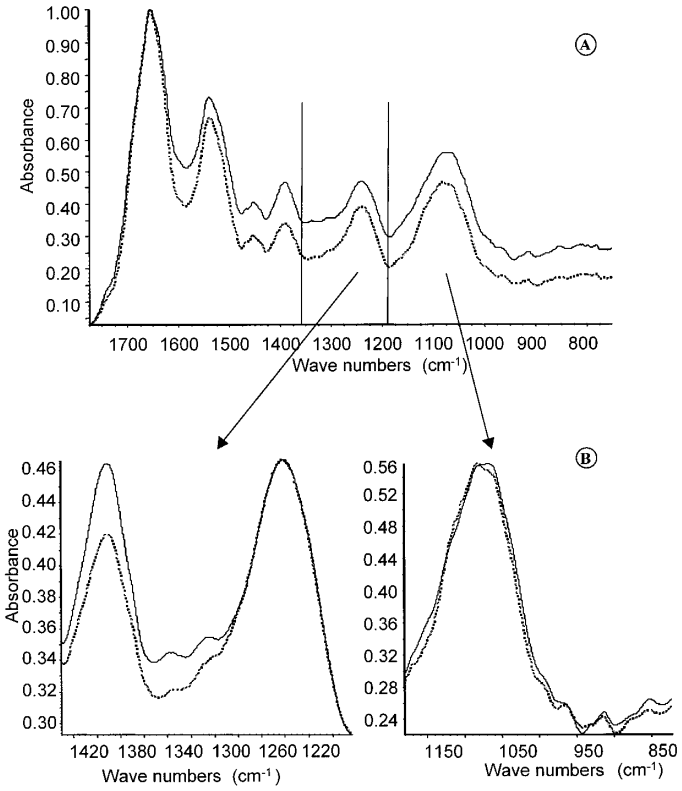

Figure 2. A: Spectra of the CNRZ reference strain (—) *Lc. lactis* ssp. *lactis* CNRZ142 and (·····) *Lc. lactis* ssp. *cremoris* CNRZ105; **B:** Zoom of 2 spectral areas from the full spectra.

Figure 2. A : Spectres des souche de référence CNRZ (—) *Lc. lactis* ssp. *lactis* CNRZ142 et (·····) *Lc. lactis* ssp. *cremoris* CNRZ105 ; **B :** Zoom de 2 zones spectrales.

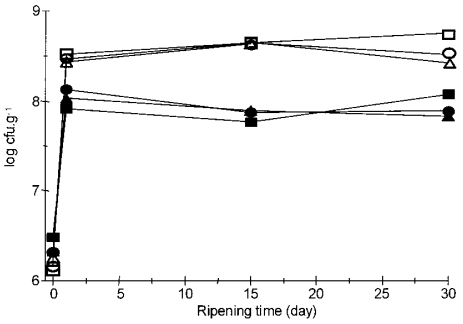

Figure 3. Evolution of *Lactococcus* flora during cheese ripening. Cheeses made with the S_A starter culture (□ series 1, ○ series 2, △ series 3); cheese made with the S_B starter culture (■ series 1, ● series 2, ▲ series 3).

Figure 3. Évolution de la flore lactocoque au cours de l'affinage des fromages. Fromages obtenus à partir du levain S_A (□ série 1, ○ série 2, △ série 3), Fromages obtenus à partir du levain S_B (■ série 1, ● série 2, ▲ série 3).

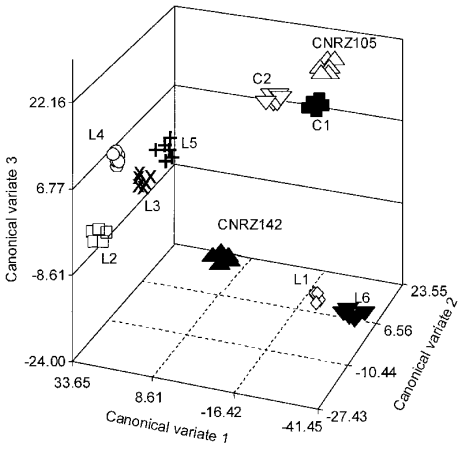

Figure 4. Discrimination of the reference strains in the first 3 canonical variates. *Lc. lactis* ssp. *lactis* (L1, L2, L3, L4, L5, L6 and CNRZ142), *Lc. lactis* ssp. *cremoris* (C1, C2 and CNRZ105).

Figure 4. Discrimination des souches de référence en fonction des 3 premières variables canoniques. *Lc. lactis* ssp. *lactis* (L1, L2, L3, L4, L5, L6 et CNRZ142), *Lc. lactis* ssp. *cremoris* (C1, C2 et CNRZ105).

Table I. Mahalanobis distances between groups in the reference strains.

Tableau I. Distances de Mahalanobis entre des groupes constitués par les souches de référence.

	CNRZ105	CNRZ142	L1	L2	L3	C1	L4	L5	L6	C2
CNRZ105	0									
CNRZ142	65	0								
L1	48	77	0							
L2	72	54	76	0						
L3	66	40	75	40	0					
C1	28	50	51	62	51	0				
L4	68	45	79	33	25	56	0			
L5	53	46	68	34	34	46	26	0		
L6	46	58	41	74	72	74	76	62	0	
C2	41	44	63	56	43	24	52	39	51	0

distances higher than 62, differentiating this group from the other *Lc. lactis* ssp. *lactis* strain.

Figure 5 presents, for each cheese and each starter culture, a breakdown of the *Lactococcus* population according to the strain identified using the discriminant model. The proportions of the 3 different strains in S_A cheeses remained relatively constant during the ripening period except for *Lc. lactis* ssp. *cremoris* (C1 strain), which did not grow in the milk or in the cheese. In S_B cheeses only 2 *Lc. lactis* ssp. *lactis* strains grew in the cheese, with L4 being dominant

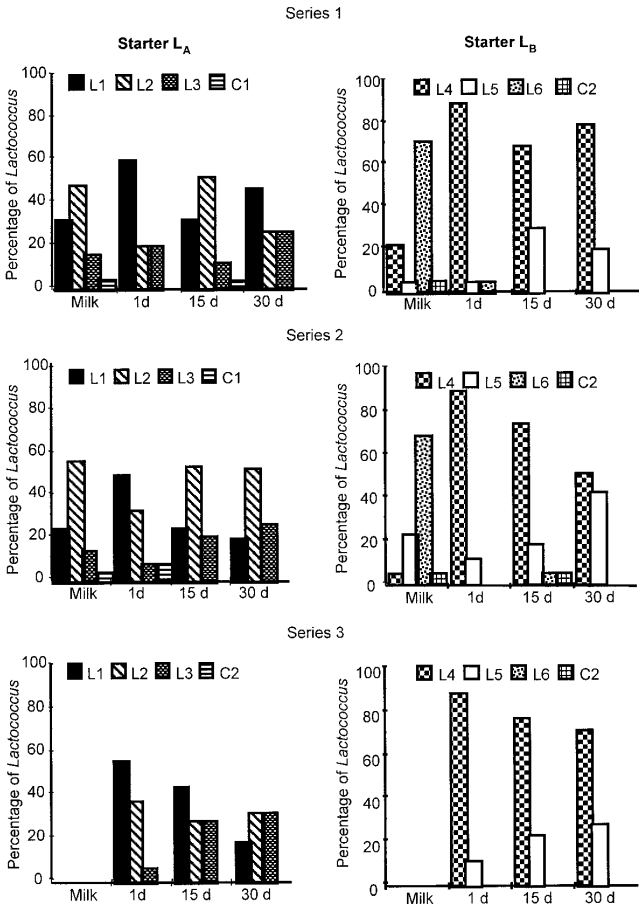

Figure 5. Proportion of the different *Lactococcus* strains during the ripening of the experimental cheeses. Starter L_A (■ L1 ▧ L2 ▩ L3 ▤ C1), starter L_B (▩ L4 □ L5 ▩ L6 ▦ C2).

Figure 5. Proportion des différentes souches de *Lactococcus* au cours de l'affinage des fromages expérimentaux. Levains L_A (■ L1 ▧ L2 ▩ L3 ▤ C1), levain L_B (▩ L4 □ L5 ▩ L6 ▦ C2).

although this strain was present in low proportions in the inoculated milk. Indeed, L6, which was present in higher proportions in the milk, disappeared in the cheese at an early stage of ripening. Similarly, in S_A cheeses the *Lc. lactis* ssp. *cremoris* (C2) grew in very low proportions in the milk and in the cheese. Since only about 1% of the colonies observed on the TSE agar plate were assumed to be *Lc. lactis* ssp. *cremoris*, all the colonies of *Lc. lactis* ssp. *cremoris* were harvested and analysed systematically to check the ability of the method to discriminate the isolate at the subspecies level. As a consequence, the proportion of the other strains was slightly underestimated by our procedure.

For this study, 530 isolates were analysed by infrared spectroscopy, and only 34 isolates (6.4%) needed further confirmation. Among these 34 isolates, 27 were reanalysed because the duplicates were

allocated to different strains of the model; however, after a second analysis, 25 of these 27 isolates were then correctly identified with only 2 remaining unidentified. The remaining 7 isolates were allocated to strains which were not used in the manufacture of the cheeses. A second analysis confirmed these 7 results.

Figure 6 shows the evolution of the *Lactococcus* flora in the control cheese made according to the same process and using the S_B culture. As with the experimental cheeses, this figure shows the prevalence of one strain, L5, over the others during the ripening period and confirms the absence or the low level of *Lc. lactis* ssp. *cremoris*. However, L4 was dominant in the experimental cheeses and L5 in the control cheese.

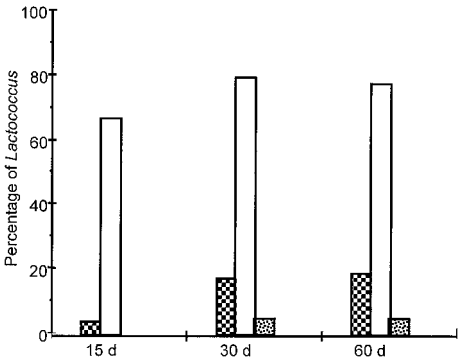

Figure 6. Proportion of the different *Lactococcus* strains during the ripening of the control cheeses (L4 L5 L6 C2).

Figure 6. Proportion des différentes souches de *Lactococcus* au cours de l'affinage du fromages de référence (L4 L5 L6 C2).

4. DISCUSSION

The larger scattering of the *Lc. lactis* ssp. *lactis* group compared to the *Lc. lactis* ssp. *cremoris* in the discriminant analysis results from the larger number of strains, i.e., 7 versus 3, but also very likely because of the wide biodiversity of the *Lc. lactis* ssp. *lactis* group. Tailliez et al. [13] showed that *Lc. lactis* ssp. *lactis* could be divided into 2 sub-groups according to their phenotypic and genetic characteristics. Indeed, the infrared spectrum provides information on the total composition of the bacterial cells. Although the assignment of some infrared regions has not been fully elucidated, it has nonetheless been established that the infrared spectrum reflects the composition of the cell membrane and cytoplasm, including nucleic acids [9]. If we bear in mind that the infrared spectra were recorded on biofilms prepared from cells resuspended in water, we may assume that because of cell membrane disruption, the information provided by the IR spectrum is representative of all the bacterial cell components. The good discrimination among the reference strains used to set up the model allows the identification of the isolates; the reliability of this method was confirmed by the similar results obtained within the series of 3 cheeses. The specificity of the model led to the correct identification of 99.6% of the 530 isolates obtained from the 7 cheeses analysed in the study. Of the 7 isolates which could not be allocated to strains from the starter culture used in the cheese making, 4 were isolated from the same S_A cheese. Concerning the 2 unidentified isolates, the spectra could not be clearly assigned to either reference strains of the model. These strains may correspond to contamination during cheese making, or the sampling procedure. We should keep in mind that FTIR identification is based on the similarity between spectra; therefore an unknown spectrum is allocated to that which is the closest in the model. In this case, in spite of good repeatability, each replicate spectrum was allocated to different strains, as if the model were unable to identify it. A correct strain identification of an unknown sample is possible only if it has been previously included in the model.

Whatever the starter culture utilised, we never found any growth of *Lc. lactis* ssp. *cremoris* during ripening. These results were

confirmed by the arginine-dihydrolase test carried out on each colony (data not included). The relative stability in the proportions of the strains of *Lc. lactis* ssp. *lactis* in S_A cheeses contrasts with the predominance of one strain in the S_B cheeses. In the case of the S_B starter, the L4 strain is the main strain identified of the *Lactococcus* flora in the experimental cheeses, whereas the L5 strain was almost the only strain in the control cheese. The prevalence of one of these 2 strains could be due to the gross composition of the cheese, mainly the moisture and the salt content. Indeed, the experimental cheeses had a higher moisture and lower salt in water content than the control cheese: respectively about 56 to 58% and 2.4 to 3.0% versus 39% and 5.68%. For both types of cheeses we can note that only the 2 strains, L4 and L5, were present throughout ripening. We assume that the third strain of *Lc.* ssp. *lactis* of the S_B culture, L6, did not grow in any cheese although it constituted about 70% of the total *Lactococcus* flora in the milk.

5. CONCLUSION

This study has shown that the specificity of the infrared spectra of bacteria allowed the identification of *Lactococcus* strains during cheese ripening. FTIR spectroscopy, which still depends on the microbiological procedures used to isolate a micro-organism, is a method that is reliable and easy to perform for the follow-up of the growth of starter cultures at strain level during maturation.

ACKNOWLEDGEMENTS

The authors thank M.H. Duployer for technical assistance, G. Duboz for the cheese making and R. Grappin for his critical reading of the manuscript.

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