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Original article

Genotypic characterisation of the dynamics of the lactic acid bacterial population of Comté cheese

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Abstract – Species and strains of lactic acid bacteria (LAB) were tracked within four commercial Comté cheeses manufactured in three factories by genetic characterisation of isolates at nine stages of cheese-making and ripening. They were also tracked in the corresponding raw milks and starter cultures. Ten species were identified: *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii, Enterococcus* sp., *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus acidilactici* and *Lactobacillus zeae*. The first four species were found to originate from the starter cultures, whereas the next three originated from raw milk. The first six species were dominant and were each represented in each cheese by eight dominant and up to 42 subdominant strains. *Lb. paracasei* was the species exhibiting the most strain diversity, from 11 to 15 different strains per cheese, followed by *Lb. rhamnosus*, from 1 to 7, and all the other species, from 1 to 5. Growth kinetics for the dominant species and strains could be obtained. Patterns of dominant LAB strains, but not species, and LAB dynamics were cheese-specific. The lactic acid microflora was found to be complex within each cheese in terms of number of different species and of different growth kinetics.

Comté cheese / lactic acid bacteria / population dynamics / strain diversity / species diversity

Résumé – Caractérisation génotypique de la dynamique des populations de bactéries lactiques dans les fromages de Comté. Les espèces et souches de bactéries lactiques ont été suivies dans quatre fromages commerciaux de Comté fabriqués dans trois fromageries différentes en caractérisant génétiquement des isolats à neuf stades de fabrication et d'affinage. Elles ont été aussi suivies dans les laits crus et les cultures de levains correspondants. Dix espèces ont été identifiées : *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii, Enterococcus* sp., *Lactobacillus paracasei*, *Lactobacillus rhamnosus*, *Lactobacillus fermentum*, *Lactobacillus plantarum*, *Pediococcus acidilactici* et *Lactobacillus zeae.* Les quatre premières espèces ont été apportées par les cultures de levains, alors que les trois suivantes l'ont été par le lait cru. Les six premières espèces étaient dominantes et chacune représentée dans chaque fromage par huit souches dominantes et jusqu'à 42 souches sous-dominantes. *Lb. paracasei* était l'espèce présentant la plus grande diversité de souches, avec de 11 à 15 souches différentes par fromage, suivie par *Lb. rhamnosus*, avec de 1 à 7 souches, et les autres espèces, avec de 1 à 5 souches. Des cinétiques de croissance ont pu être établies pour les souches et espèces dominantes. Le profil des souches dominantes, mais non des espèces, et la dynamique des bactéries lactiques étaient spécifiques à

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chaque fromage. La flore lactique était complexe pour chaque fromage, en terme de nombre d'espèces présentes et de cinétiques de croissance différentes observées.

Fromage de Comté / bactérie lactique / dynamique bactérienne / diversité des souches / diversité des espèces

1. INTRODUCTION

Lactic acid bacteria (LAB) are dominant in the microflora of all cheese varieties and are of major importance in this respect in the formation of the properties of mature cheeses. In each cheese, the microflora is generally complex, composed of different species in a dynamic state throughout cheese-making and ripening. The species profile is generally comparable for all the cheeses within a same variety, but the dynamics of each species can vary. The complexity is greater in long-ripened cheeses by the presence in the dominant flora of non-adjunct species composed themselves of different strains. This was shown for mesophilic lactobacilli in commercial Cheddar or Comté cheeses [3, 14] or for thermophilic lactobacilli in commercial Comté cheeses [4]. The complexity is further enhanced by the specific pattern of the non-adjunct strains in each cheese within a variety [3, 4, 14].

Comté cheese is a traditional hard-cooked ripened cheese manufactured in relatively small factories from raw milk. In this type of cheese the internal part represents the major part and the microflora of this group of cheese have been described during cheese-making and/or ripening in various varieties [5, 7, 15, 22]. Lactic acid bacteria and propionibacteria were the two major groups of microflora detected in Comté cheese [16]. LAB species identified in Comté cheese were the same as in other cooked cheese varieties: *Streptococcus thermophilus*, *Lactobacillus helveticus*, *Lactobacillus delbrueckii* spp. *lactis*, *Lactobacillus fermentum*, *Lactobacillus paracasei, Lactobacillus rhamnosus*, enterococci sp. and pediococci sp.

The elucidation of the role of microflora requires the description of the microflora at pertinent levels in order to explain the constant and variable properties of mature cheeses within each cheese variety and between different varieties. Variability of microflora between cheeses within a variety has been found at the strain level but not at the species level, and has been related to the presence of non-adjunct strains provided by the milk, environment or natural starter [3, 4, 9, 14]. Most studies on cooked cheeses have reported the inadequacy of counts per gram of cheese after cultivation on different media to estimate directly LAB species counts because of the non-selectivity of the media [6–8, 15, 16, 22]. The reliable assignment of isolates at the strain and species levels by molecular methods has recently allowed the description of the diversity, and in some cases the identification of the source, of mesophilic lactobacilli. This was the case for mesophilic lactobacilli isolated from Cheddar and Comté cheeses [3, 9, 13] and for thermophilic lactobacilli isolated from other Comté cheeses [4]. This approach was first applied to mesophilic lactobacilli isolated from one culture medium and from two commercial Comté cheeses, manufactured at the same time in two different factories but ripened under the same conditions [3]. In the present study the same approach was extended to LAB isolated from four culture media usually used to track a wide range of starter and non-starter LAB in different cooked and ripened cheese varieties [7, 15, 16, 19, 22] and was applied to LAB isolated from two other commercial Comté cheeses manufactured 18 months later than the cheeses studied in [3]. The cheeses originated from two different factories, one of them being the same as in the former study,

and they were ripened under the same conditions.

This paper focuses on the LAB strains and species collected in the four commercial Comté cheeses as described above regarding their source, their number, their taxonomy and their cell density according to cheese-making and ripening times. It completes the results already presented for mesophilic lactobacilli in two of the cheeses [3] by presenting their dynamics. The complexity and diversity of LAB microflora within each cheese and among Comté cheeses is discussed.

2. MATERIALS AND METHODS

2.1. Samples

Four Comté cheeses were manufactured in three different factories (1, 2, and 3) at two different times, cheeses A and B in factories 1 and 2, and 18 months later, cheeses C and D in factories 1 and 3, according to each factory's usual practices. Cheeses A and B correspond to the 1b and 2b cheeses described and analysed in [3]. The four cheeses were ripened according to similar ripening schemes at 13 °C for 6 to 7 weeks, at 17 °C for 6 to 7 weeks and at 6 °C until their optimal ripening time, from 5.6 to 9.3 months. Samples were obtained from the milks and the starter cultures which were used for cheese manufacture, from the curd, and from the cheeses at day 1, 7, 21, 42 (cheeses C and D) or 49 (cheeses A and B), 63, 91, 110 (cheeses C and D) or 122 (cheeses A and B), 182 (cheeses C and D), and at their optimal ripening time (cheeses A, B, C, and D).

2.2. Enumeration and isolation of lactic acid bacteria

Milks, starter cultures, curds and cheeses were sampled and isolates were recovered as described by Berthier et al. [3]. Samples from cheeses A and B and the corresponding milks and starter cultures were only inoculated on FH or MRS plates (5 d, 20 °C, anaerobiosis) [3, 18]. Samples from cheeses C and D and the corresponding milks and starter cultures were inoculated on FH-20 (5 d, 20 °C, anaerobiosis) to enumerate mesophilic and heterofacultatively heterofermentative lactobacilli, MRS-45 (3 d, 4 °C, pH 5.5, anaerobiosis) for thermophilic lactobacilli, and M17-45 or -20 (2 d, 45 °C or 20 °C/40 $g \cdot L^{-1}$ of nalidixic acid, aerobiosis) for thermophilic streptococcocci and lactococci, respectively [10, 18, 21]. Isolates, usually 20, were recovered from C and D curd and cheese samples cultivated on each medium at the stages indicated in Table I. Isolates were recovered from 4 milks and 11 culture starter samples as indicated in Table II. A total of 1833 isolates was analysed.

2.3. DNA extraction

DNA was extracted as previously described [3].

2.4. Strain typing

Rep-PCR was performed as previously described [3] and applied to all isolates. Isolates exhibiting profiles with 88% or more similarity were considered to be the same strain.

2.5. Species identification

PCR was applied to isolates representative of each Rep-PCR profile. PCR specific for *Lb. paracasei*, *Lb. zeae*, *Lb. rhamnosus*, *Lb. plantarum*, *Lb. curvatus*, *Lb. delbrueckii*, *Lactococcus lactis* and *St. thermophilus* was performed as previously described [1–3, 12, 20]. A newly-designed primer from the long 16S-23S intergenic sequence of *Lb. helveticus*, CCCCAAGGTC TTTTATTTC, was used for *Lb. helveticus*-specific PCR in the conditions described by Berthier et al. [2], except that annealing was performed at 61 °C for 0 s. In this way, *Lb. helveticus* strains can be differentiated from the very closely related *Lb. gallinarum* strains.

Table I. Counts of bacterial species (counts at time 0 are given only for strains present in cheese; they were obtained by multiplying tenfold the counts in milk to take account of the concentration due to mouldind and pressing); in bold, counts of dominant species; painted grey, counts were under the indicated counts (detection limit).

Cheese	Isolation medium	Species	Bacterial counts in cheese (cfu·g^{-1} of cheese)									
					Preripening			Warm room		Cold room		
		Ripening temperature (°C)			37	13	13	17	17	6	6	6
		Ripening time (d)	0	0.18	1	7	21	49	63	91	122	280
	FH 20/MRS 20	***Lb. rhamnosus***	1.02E+04	6.30E+03	1.34E+04	6.24E+05	**2.28E+07**	**7.20E+07**	**8.80E+07**	**4.50E+07**	**8.40E+07**	*1.00E+06*
	FH 20/MRS 20	***Lb. paracasei***	6.80E+03	1.17E+04	5.61E+03	1.66E+05	**3.42E+07**	**1.08E+08**	**7.20E+07**	**5.50E+07**	**5.60E+07**	**2.10E+07**
A	**M17 45**		N.D.	N.D.	N.D.	7.20E+07	7.60E+07	8.00E+07	N.D.	N.D.	N.D.	N.D.
	MRS 45		N.D.	N.D.	N.D.	3.50E+08	8.40E+07	7.80E+07	N.D.	N.D.	N.D.	N.D.
		Ripening time (d)	0	0.18	1	7	21	49	63	91	122	168
	FH 20/MRS 20	***Lb. rhamnosus***	1.00E+02	N.D.	*1.10E+03*	*1.90E+03*	2.40E+05	1.30E+06	**1.31E+07**	**7.50E+06**	**2.30E+07**	*2.00E+06*
	FH 20/MRS 20	***Lb. paracasei***	1.39E+03	N.D.	*1.10E+03*	*1.90E+03*	2.16E+06	**2.47E+07**	**7.40E+07**	**1.43E+08**	**9.20E+07**	**3.90E+07**
B	**FH 20**	***Lb. fermentum***	3.20E+02	N.D.	2.30E+04	3.76E+04	*1.20E+05*	*1.30E+06*	*4.35E+06*	*7.50E+06*	*5.75E+06*	*2.00E+06*
	FH 20/MRS 20	***Lb.* sp.**	*0.08E+00*	N.D.	*1.10E+03*	*1.90E+03*	*1.20E+05*	*1.30E+06*	*4.35E+06*	*7.50E+06*	*5.75E+06*	*2.00E+06*
	M17 45		N.D.	N.D.	N.D.	2.50E+08	1.20E+08	8.40E+07	N.D.	N.D.	N.D.	N.D.
	MRS 45		N.D.	N.D.	N.D.	1.40E+08	1.40E+07	3.20E+07	N.D.	N.D.	N.D.	N.D.

Table I. Continued.

		Ripening time (d)	0	0.18	1	7	21	42	63	110	182	230
	FH 20/MRS 45/M17 45	***Lb. rhamnosus***	2.62E+02	1.90E+02	6.50E+02	*2.30E+03*	6.93E+06	**1.10E+08**	**9.70E+07**	**3.69E+07**	**1.25E+07**	**7.96E+06**
	FH 20	***Lb. paracasei***	5.29E+02	5.73E+02	4.23E+03	4.37E+04	8.15E+05	5.80E+06	*4.85E+06*	**4.10E+06**	**4.46E+06**	**1.90E+06**
	FH 20	***Lb. fermentum***	*5.00E+01*	*3.63E+01*	9.75E+02	2.30E+03	*4.08E+05*	*5.80E+06*	*4.85E+06*	*2.05E+06*	*8.50E+05*	*4.75E+05*
	MRS 45/M17 45	***Lb. zeae***	N.D.	*1.65E+05*	*4.00E+07*	*2.75E+07*	*7.50E+06*	8.50E+06	N.D.	1.40E+06	N.D.	7.20E+05
	MRS 45	***Pd. acidilactici***	N.D.	*1.65E+05*	*4.00E+07*	*2.75E+07*	*7.50E+06*	*4.62E+06*	N.D.	N.D.	N.D.	7.20E+05
	FH 20	***Lb. plantarum***	*5.00E+01*	*3.63E+01*	6.50E+02	*2.30E+03*	*4.08E+05*	*5.80E+06*	*4.85E+06*	*2.05E+06*	*8.50E+05*	*4.75E+05*
C	**M17 45**	***Ent* sp.**	9.90E+02	**9.60E+06**	*1.45E+07*	*2.65E+06*	*5.75E+05*	*4.25E+06*	N.D.	N.D.	N.D.	*3.40E+05*
	MRS 45	***Lb. helveticus***	**1.10E+06**	**1.16E+06**	*4.00E+07*	*2.75E+07*	*7.50E+06*	*4.62E+06*	N.D.	*1.40E+06*	N.D.	*3.60E+05*
	MRS 45 (M17 45)	***Lb. delbrueckii***	**1.30E+06**	**2.15E+06**	**8.00E+08**	**5.50E+08**	**1.43E+08**	**9.23E+07**	N.D.	*1.40E+06*	N.D.	3.60E+05
	M17 45 (M17 20)	***St. thermophilus***	**1.07E+07**	**2.24E+07**	**2.58E+08**	*2.65E+06*	*5.75E+05*	*4.25E+06*	N.D.	N.D.	N.D.	*3.40E+05*
	M17 20	***Lc. lactis***	8.30E+04	*8.00E+04*	*4.70E+04*	*4.25E+03*	*4.80E+05*	N.D.	N.D.	N.D.	N.D.	N.D.
		Ripening time (d)	0	0.18	1	7	21	42	63	110	182	230
	FH 20/MRS 45/M17 45	***Lb. rhamnosus***	*1.20E+02*	5.94E+01	1.18E+03	*2.25E+03*	*4.50E+05*	1.59E+06	**7.48E+06**	2.64E+06	**9.50E+05**	2.34E+05
	FH 20	***Lb. paracasei***	3.75E+02	8.37E+02	4.70E+02	4.05E+04	**1.13E+07**	**5.60E+07**	**1.40E+08**	**7.47E+07**	**8.00E+06**	**1.66E+07**
	(FH 20)	***Lb. fermentum***	1.20E+02	*4.50E+01*	3.06E+03	4.50E+03	*4.50E+05*	*2.80E+06*	*7.00E+06*	*4.15E+06*	*4.00E+05*	*8.75E+05*
	MRS 45	***Lb. fermentum***	N.D.	*2.25E+04*	6.30E+05	*2.10E+05*	*1.15E+05*	*1.65E+05*	*2.20E+05*	N.D.	N.D.	*2.15E+04*
	MRS 45	***Lb. zeae***	N.D.	*2.25E+04*	*1.25E+07*	*2.10E+05*	*1.15E+05*	*1.65E+05*	*2.20E+05*	N.D.	N.D.	2.15E+04
	M17 45	***Pd. acidilactici***	N.D.	*1.75E+06*	*9.00E+06*	*7.00E+06*	*6.00E+05*	*3.00E+05*	*3.25E+05*	*1.55E+05*	*5.00E+04*	1.56E+05
D		***Lb. plantarum***	*1.20E+02*	*4.50E+01*	*2.35E+02*	*2.25E+03*	*4.50E+05*	*2.80E+06*	*7.00E+06*	*4.15E+06*	*4.00E+05*	*8.75E+05*
	M17 45/M17 20	***Ent* sp.**	4.40E+03	**1.75E+06**	*9.00E+06*	*7.00E+06*	6.00E+05	3.00E+05	3.25E+05	*1.55E+05*	*5.00E+04*	*1.95E+04*
	MRS 45	***Lb. helveticus***	**1.50E+05**	3.60E+05	**1.23E+08**	6.30E+05	1.15E+05	1.65E+05	2.20E+05	N.D.	N.D.	*2.15E+04*
	MRS 45	***Lb. delbrueckii***	**3.10E+05**	9.00E+04	**1.02E+08**	2.94E+06	**2.30E+06**	1.90E+06	6.60E+05	N.D.	N.D.	*2.15E+04*
	M17 45 (M17 20)	***St. thermophilus***	**3.00E+06**	**3.33E+07**	**1.80E+08**	**1.40E+08**	**1.14E+07**	**5.40E+06**	*3.25E+05*	*1.55E+05*	*5.00E+04*	*1.95E+04*
	M17 20	***Lc. lactis***	3.30E+06	*2.25E+03*	*6.00E+03*	*3.55E+03*	*4.50E+05*	N.D.	N.D.	N.D.	N.D.	N.D.

Table II. Bacterial counts in milks and starter cultures (cfu·mL^{-1}).

Culture medium	Raw milk	Renneting milk	Starter culture		
	A	A	A1	A2	A3
MRS 20	2.80E + 03	1.80E + 03	3.00E + 01	7.80E + 02	4.50E + 06
FH 20	6.60E + 02	3.60E + 03	1.00E + 01	2.50E + 02	2.90E + 03
	B	B	B1	B2	
MRS 20	>10000	9.70E + 04	6.00E + 02	3.00E + 01	
FH 20	1.60E + 02	2.70E + 02	2.00E + 01	<10	
	C	C	C1	C2	C3
MRS 45	1.50E + 01	3.00E + 05	6.70E + 05	1.30E + 07	6.00E + 08
FH 20	1.50E + 02	2.10E + 02	1.10E + 04	<25	1.50E + 01
M17 45	9.00E + 02	1.40E + 06	4.80E + 08	4.90E + 07	1.60E + 08
M17 20	1.20E + 03	3.00E + 04	8.30E + 06	9.00E + 06	1.00E + 06
	D	D	D1	D2	D3
MRS 45	4.30E + 04	6.00E + 04	1.10E + 06	3.30E + 07	2.20E + 07
FH 20	4.40E + 02	5.00E + 02	2.00E + 01	<5	<5
M17 45	1.20E + 06	1.30E + 06	6.60E + 02	3.00E + 08	1.00E + 03
M17 20	3.10E + 05	3.40E + 05	3.50E + 08	1.00E + 05	7.60E + 02

Analysed samples.

Strains were presumably assigned to *Enterococcus* sp., *Lb. fermentum* and *Pd. acidilactici* if their Rep-PCR profiles exhibited 40% or more homology with the profiles of the reference strains of these genus or species. Morphology, growth temperature and type of fermentation were used to support the assignation.

2.6. Strain and species counts

Counts for a particular strain were estimated by multiplying the percentage of this strain in the isolates by a total count of bacteria on the medium from which the isolates were recovered. Each species count was estimated by adding the counts of all strains assigned to the same species in a sample.

3. RESULTS

3.1. Selectivity of media

All media allowed the cultivation of other species than those for which they were specified. FH-20 medium allowed the enumeration of the cultivable cells of the mesophilic and facultatively heterofermentative lactobacilli *Lb. paracasei*, *Lb. plantarum* and *Lb. rhamnosus* species, but also part of the thermophilic and obligatory heterofermentative *Lb. fermentum* species. Both MRS-45 and M17-45 culture media allowed the enumeration of the cultivable cells of thermophilic lactobacilli and streptococci species, respectively, but also exhibited *Lb. rhamnosus* species (which could also be enumerated on FH-20 medium) and *Lb. zeae* and *Lb. fermentum* species (which

could not be enumerated on FH-20 because of their low abundance and/or poor growth on this medium).

Similar counts were obtained for *Lb. rhamnosus* cells on three different media, FH-20, MRS-45 and M17-45, when counts allowed an enumeration on the different media (Tab. I). Counts higher by one to two log were obtained for *St. thermophilus* on M17-45 versus M17-20 medium, for *Lb. delbrueckii* on MRS-45 versus M17-45 medium, and for *Lb. fermentum* on MRS-45 versus FH-20 medium. Similar counts for *Enterococcus* sp. were obtained in milk C on M17-45 and M17-20, but this species could only be enumerated on M17-45 in curds of C and D, although its counts allowed its enumeration on M17-20.

3.2. Species of LAB in Comté cheese

3.2.1. Diversity

Ten species of lactic acid bacteria were identified in the microflora of cheeses C and D in the curd and throughout ripening when MRS, M17 and FH culture media were used (Tab. I). Nine species were common to both cheeses. *Lb. rhamnosus*, *Lb. paracasei*, *Lb. helveticus*, *Lb. delbrueckii, Enterococcus* sp. and *St. thermophilus* were the most abundant in both cheeses, and *Lb. zeae*, *Lb. fermentum, Lb. plantarum* and *Pd. acidilactici* were less abundant or undetectable in both cheeses. *Lc. lactis* was never recovered. *Lb. paracasei* and *Lb. rhamnosus* were also the most abundant mesophilic lactobacilli in cheeses A and B. A non-identified species of obligatory heterofermentative and mesophilic lactobacilli was detected once in cheese B.

3.2.2. Dynamics

The pattern of dominant species, thermophilic and mesophilic species, varied in the same way within each cheese as a function of time. Thermophilic species, *Lb. helveticus*, *Lb. delbrueckii, Enterococcus* sp. and/or *St. thermophilus,* were the predominant cultivable LAB from cheese-making up until 21 d of ripening in the two cheeses in which they were looked for (C and D), whereas mesophilic species, *Lb. paracasei* and/or *Lb. rhamnosus,* were the predominant cultivable LAB from 21 or 49 d to the end of ripening in the four cheeses. Each dominant thermophilic LAB species, except *Enterococcus* sp., generally reached their highest cell density (log 8 to log 9 cfu·g^{-1}) after one day from initial counts between log 5 and log 6 cfu·g^{-1}. *Enterococcus* sp. was detected only in the curd at log 7.0 and 6.2, in cheeses C and D, respectively. Each dominant mesophilic LAB species generally reached their highest cell density after 21 or 49 d of ripening (log 7 to log 8 cfu·g^{-1}), from initial counts between log 2 and log 4 cfu·g^{-1}. Mesophilic and thermophilic LAB were at similar counts at 21/49 d of ripening (log 7 to log 8 cfu·g^{-1}, depending on the cheese).

The pattern of subdominant species varied in the same way within each cheese as a function of time. *Lb. plantarum* was detected only in one of the four cheeses and only during cheese-making, whereas *Lb. fermentum* was detected in three of the four cheeses during cheese-making and up until 7 d of ripening. *Lb. zeae* and *Pd. acidilactici* were detected in the two cheeses in which they were looked for at the end of ripening with counts 2 log lower than dominant mesophilic LAB. *Lb. fermentum* was at its highest density at 7 d of ripening and then declined and became undetectable. *Lb. zeae* exhibited the same dynamics as *Lb. rhamnosus* but at a one log lower cell density. *Pd. acidilactici* was detected at the end of ripening at log 5 cfu·g^{-1}.

The pattern of dominant species also varied from cheese to cheese. *St. thermophilus* was the most dominant thermophilic species in cheese D whereas *Lb. delbrueckii* was the most dominant thermophilic species in cheese C. *Lb. rhamnosus* was the most dominant mesophilic species in cheese C whereas *Lb. paracasei* was the most dominant mesophilic species in cheeses B and D, and both species co-dominated in cheese A.

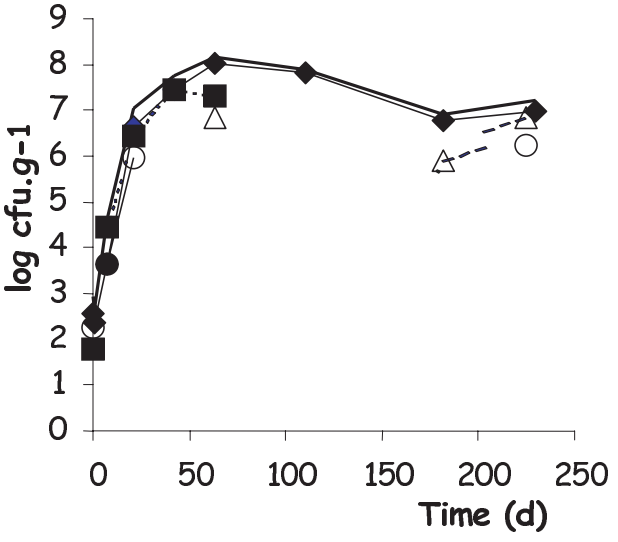

Figure 1. Counts of *Lb. paracasei* species (—) and 4 *Lb. paracasei* strains (◆, ■, ○, △) in cheese D.

The kinetics of growth of each dominant thermophilic LAB species varied from cheese to cheese. *St. thermophilus* reached the same cell density at day one from similar counts in the curd, but its number decreased faster in cheese C than in cheese D. A similar number of cells for each *Lb. helveticus* and *Lb. delbrueckii* species were inoculated but *Lb. helveticus* counts were at least 1.3 log lower in cheese C at day one, and similar in cheese D. *Lb. delbrueckii* cell density increased by 3 log during the first day in both cheeses, but decreased by 1.5 log in cheese D from day one to day seven, while remaining the same in cheese C.

Lb. helveticus counts decreased faster than *St. thermophilus* counts in both cheeses C and D, with at least 1 log less in cheese C at day one and 2.3 log less in cheese D at day seven.

3.3. Strains of lactic acid bacteria in Comté cheese

3.3.1. Diversity

As shown in Table III, one to 15 strains of each species were found in each cheese, with *Lb. paracasei* exhibiting the greatest diversity. Only seven LAB strains were common to cheeses C and D out of a total of 61: one strain of *St. thermophilus*, one strain of *Enterococcus* sp., one strain of *Lb. helveticus*, two strains of *Lb. paracasei* and two strains of *Lb. rhamnosus*. No mesophilic lactobacilli strains were common to cheeses A and B or to cheeses A and C.

3.3.2. Dynamics

Usually only one to two strains of each dominant species represented 80 to 100% of the species isolates from each cheese and reached a cell density of log 6.7 cfu·g^{-1} or more (Tab. III). Figure 1 shows the growth kinetics for 4 of the 15 *Lb. paracasei* strains from cheese D and illustrates the diversity of kinetics which was observed in one cheese for strains belonging to one species. Out of the seven common strains between cheeses C and D, four did not exhibit the same growth kinetics in each cheese, one *St. thermophilus*, two *Lb. paracasei* and one *Lb. rhamnosus* strains, three of them being dominant only in one of the cheeses, whereas one subdominant *Lb. rhamnosus* strain exhibited similar growth kinetics. Two strain kinetics, one of

Table III. Survey of LAB strains in Comté cheeses.

Cheese	Species	Strain				Isolates
		Total number	Number of dominant strains	Number of strains with known sources*	% of species isolates/each strain*" (isolation medium)	Total number per cheese#
A	***Lb. rhamnosus***	1	**1**	1	**100** (MRS 20)	228
	Lb. paracasei	12	**2**	4	**56, 24**, 5 (MRS 20)	
B	***Lb. rhamnosus***	1	**1**	1	**100** (MRS 20)	180
	Lb. paracasei	13	**5**	5	**28, 20, 11, 10, 9** (MRS 20)	
	Lb. fermentum	3	**1**	1	**87** (FH 20)	
	Lb. sp.	1				
C	***Lb. rhamnosus***	5	**1**	1	**95** (FH 20)	720
	Lb. paracasei	11	**2**	4	**25, 15** (FH 20)	
	Lb. fermentum	1	?¤	0		
	Lb. zeae	3	?	0		
	Pd. acidilactici	2	?	0		
	Lb. plantarum	1	?	0		
	***Enterococcus* sp.**	1	**1**	*0*		
	Lb. helveticus	1	**1**	*1*	***100*** (MRS 45)	
	Lb. delbrueckii	1	**1**	*1*	***100*** (MRS 45)	
	St. thermophilus	3	**2**	*3*	***47, 37***, 16 (M17 45)	
D	***Lb. rhamnosus***	7	**2**	0	**50, 41**, 4 (M17 45)	705
	Lb. paracasei	15	**2**	1	**56, 21** (FH 20)	
	Lb. fermentum	3	?	0		
	Lb. zeae	1	?	0		
	Pd. acidilactici	4	?	0		
	***Enterococcus* sp.**	1	**1**	*0*		
	Lb. helveticus	2	**1**	*1*	***100*** (MRS 45)	
	Lb. delbrueckii	3	**1**	*1*	***91*** (MRS 45)	
	St. thermophilus	1	**1**	*1*	***98*** (M17 45)	

*Authenticated sources: starter culture, in italics; milk, normal. " Dominant strains in bold; # including isolates from milk and starter cultures; ¤ could not be determined.

an *Enterococcus* sp. and one of a *Lb. helveticus* strain, were difficult to compare because cell densities were available at only two stages.

3.3.3. Origin

The origin could be established at best for only some strains of each species.

Fourteen of the 51 *Lb. paracasei*, one of the 7 *Lb. fermentum* and two of the 14 *Lb. rhamnosus* cheese strains were detected in the corresponding raw milk. Ten of the 17 cheese strains detected in the milk were dominant in the cheese (Tab. III). But six *Lb. paracasei* strains detected at a similar cell density to the former in milks B and C were not detected in the corresponding cheeses, and four *Lb. paracasei* strains dominant in cheeses B and D were not detected in the corresponding milk.

The four *St. thermophilus* cheese strains, two of the three *Lb. helveticus* cheese strains, and two of the four *Lb. delbrueckii* cheese strains were detected in the corresponding starter cultures at cell densities compatible with their densities after starter addition to milk. The eight cheese strains detected in the starters were dominant in the cheese. Only one *St. thermophilus* strain was detected in the starters but not in the cheese.

The *Lb. plantarum*, *Lb. zeae*, *Pd. acidilactici* and *Lb. fermentum* cheese strains were not detected in the corresponding raw milk or starter cultures, except one *Lb. fermentum* strain of cheese B in the milk. The dominant *Enterococcus* sp. strain common to cheeses C and D was detected in the corresponding milk, but at cell densities too low regarding their cell densities in the curds (Tab. I). It is therefore likely that its source was starter culture, all the more so as it reached a high level in the whey that served as culture medium for starter strains. Two starter cultures could be the source of this strain, assuming that its growth rate was similar to those of the *Lb. delbrueckii* and *Lb. helveticus* strains until day one; starter addition could have inoculated this strain in milks C and D at a maximal cell density of log 4.5 and 4.2 cfu·g^{-1}, respectively.

4. DISCUSSION

The simultaneous tracking of many cultivable LAB at strain and species levels at nine different stages of cheese-making and ripening has provided a novel and accurate picture of the species and strains present in commercial Comté cheeses, particularly from a dynamic point of view. This work allowed to completely change the way of discribing the microflora of Comté cheese by getting a growth kinetics for each individual LAB strain and species in place of one growth kinetics, including several strains and species that changed according to the cheese-making and ripening stages. The lack of selectivity of the culture media was again largely demonstrated in this study.

Two species, *Lactobacillus zeae* and *Lactobacillus plantarum,* were newly detected in Comté cheeses. The former species has never been reported in any other cheese variety, and the second has already been reported in Cheddar cheeses [9, 14]. Eleven different LAB species were detected in Comté cheeses, with nine to ten species detected in each individual cheese. The profile of LAB species in each cheese differed only by one minor species.

All the Comté cheeses examined so far are characterised by the succession in their dominant cultivable microflora of the same four thermophilic LAB species during cheese-making and early ripening, and then the same two mesophilic LAB species during late ripening, as previously observed [4, 16]. In that respect, Comté cheeses could not be differentiated and were similar to all other cooked cheese varieties. This general pattern is very probably linked to the differences in the cell counts between LAB species in the milk at the beginning of cheese-making and to the thermal gradient applied during cheese-making. The tracking of strains in raw milks, starter cultures and cheeses allowed the determination of the source of inoculation for several cheese strains, including the majority of dominant strains in cheese. Dominant strains of *Lb. delbrueckii*, *Lb. helveticus*, *St. thermophilus* and very probably *Enterococcus* sp. thermophilic species

originated from starter cultures, whereas most dominant strains of *Lb. paracasei* and *Lb. rhamnosus* mesophilic species originated from raw milks. After the addition of starter cultures to raw milk, the thermophilic strains of the starter cultures added to the milk were present at least at two log more than the mesophilic strains, except *Lc. lactis* strains. *Lc. lactis* strains, even if inoculated from starter culture in cheese D at a similar level to thermophilic LAB, decreased by at least 3 log at pressing, certainly because of the heating at 56 °C for 25 min which preceded it. A loss of cultivability of the thermophilic species was observed during cheese-making or at the beginning of ripening, depending on the species and the cheese, which allowed the mesophilic lactobacilli to become the dominant cultivable microflora after 21/49 d of ripening. These species could respond to the exhaustion of energetic substrates [17] and/or the multiple variations in pH, NaCl concentration and temperature which occurred at that time. The loss of cultivability of *St. thermophilus* and *Lb. helveticus* species in both cheeses C and D has already been observed in experimental cooked mini-cheeses [11]. Its kinetics was similar to that of one of the eight combinations of *St. thermophilus* and *Lb. helveticus* strains previously examined [11].

The pattern of subdominant species in the cheese is probably linked to their low counts in the natural microflora of milk and to their rather thermophilic properties, opposed to the mesophilic properties and higher counts in raw milks of the two dominant species during ripening, *Lb. paracasei* and *Lb. rhamnosus*. The *Lb. fermentum*, *Lb. zeae*, and *Pd. acidilactici* subdominant species could not be detected either in the milks or starter cultures, except a *Lb. fermentum* strain which reached the highest counts in the cheese. All the same, these species may have been present in the milk, but were undetectable due to their low counts and the lack of selectivity of the media used. They were only detected occasionally in the cheese for similar reasons. *Lb. fermentum* growth exhibited the highest rate before day one, when the temperature was around 45 °C for a few hours.

One main difference between the cheeses was the growth kinetics of each dominant species which induced very different pictures of the species dynamics in each cheese. Growth kinetics were different in terms of the initial cell density, length of the latency phase, exponential growth rate, maximal cell density, and length of the stationary phase. In this respect, each Comté cheese exhibited a specific pattern of LAB species. This pattern varied between two cheeses manufactured at the same period in two different factories as well as in the two cheeses manufactured at two different periods within the same factory.

The pattern of LAB strains was also specific for each cheese. The seven strains common to cheeses C and D represented 24 and 17% of the number of strains in cheese C and D, respectively, but most of them exhibited different growth kinetics in each cheese so that the pattern of dominant strains was specific for each cheese. One or two strains per species reached levels susceptible to impact cheese properties, i.e. more than log 6 cfu·g^{-1}. In that respect, eight strains representing six LAB species could constitute the identity card of each cheese.

The tracking of LAB by genetic characterisation of isolates throughout cheese-making and ripening allowed us to get a quantitative picture of microflora dynamics at both strain and species levels. Strain composition and enumeration were essential to distinguish between cheeses. This approach allowed us to get growth kinetics for dominant strains and species, even at stages at which they were subdominant, but not for always subdominant strains and species. Such data will be very useful for understanding the dynamics of the cheese ecosystem. They already substantiate the complexity of LAB within each cheese and between cheeses. Each cheese exhibited up to ten different species, up to 40 different strains

and specific dynamics for many species. Nevertheless, diversity expressed as a number of strains should be tempered by the cell density reached by each detected strain. The different cheeses exhibited a specific composition for dominant strains and specific dynamics of their microflora. A similar approach applied to the other dominant microflora, the propionibacteria, will complete the accurate description of microflora within Comté cheese.

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REFERENCES

[1] Beimfohr C., Ludwig W., Schleifer K.H., Rapid genotypic differentiation of *Lactococcus* subspecies and biovar, Syst. Appl. Microbiol. 20 (1997) 216–221.

[2] Berthier F., Ehrlich S.D., Rapid species identification within two groups of closely related lactobacilli using PCR primers that target the 16S/23S rRNA spacer region, FEMS Microbiol. Lett. 161 (1998) 97–106.

[3] Berthier F., Beuvier E., Dasen A., Grappin R., Origin and diversity of mesophilic lactobacilli in Comté cheese, as revealed by PCR with repetitive and species-specific primers, Int. Dairy J. 11 (2001) 293–305.

[4] Bouton Y., Guyot P., Beuvier E., Tailliez P., Grappin R., Use of PCR-based methods and PFGE for typing and monitoring homofermentative lactobacilli during Comté cheese ripening, Int. J. Food Microbiol. 76 (2002) 27–38.

[5] Chamba J.-F., Delacroix-Buchet A., Berdagué J.L., Clément J.-F., Une approche globale de la caractérisation des fromages : l'exemple du fromage de Beaufort, Sci. Aliments 14 (1994) 581–590.

[6] Coppola R., Nanni M., Iorizzo M., Sorrentino A., Sorrentino E., Grazia L., Survey of lactic acid bacteria isolated during the advanced stages of the ripening of Parmigiano Reggiano cheese, J. Dairy Res. 64 (1997) 305–310.

[7] Coppola R., Nanni M., Iorizzo M., Sorrentino A., Sorrentino E., Chiavari C., Grazia L., Microbiological characteristics of Parmigiano Reggiano cheese during the cheesemaking and the first months of the ripening, Lait 80 (2000) 479–490.

[8] Coppola R., Nanni M., Succi M., Sorrentino A., Iorizzo M., Chiavari C., Grazia L., Enumeration of thermophilic lactic acid bacteria in ripened cheeses manufactured from raw milk, Milchwissenchaft 56 (2001) 140–142.

[9] Dasen A., Berthier F., Grappin R., Williams A.G., Banks J., Genotypic and phenotypic characterisation of the dynamics of the lactic acid bacterial population of adjunct-containing Cheddar cheese manufactured from raw and microfiltered pasteurised milk, J. Appl. Microbiol. 94 (2003) 595–607.

[10] De Man J.C., Rogosa M., Sharpe M.E., A medium for the cultivation of lactobacilli, J. Appl. Bacteriol. 23 (1960) 130–135.

[11] Deutsch S.M., Ferain T., Delcour J., Lortal S., Lysis of lysogenic strains of *Lactobacillus helveticus* in Swiss cheeses and first evidence of concomitant *Streptococcus thermophilus* lysis, Int. Dairy J. 12 (2002) 591–600.

[12] Drake M., Small C.L., Spence K.D., Swanson B.G., Rapid detection and identification of *Lactobacillus* sp. in dairy products by using the polymerase chain reaction, J. Food Prot. 59 (1996) 1031–1036.

[13] Fitzsimons N.A., Cogan T.M., Condon S., Beresford T., Phenotypic and genotypic characterization of non-starter lactic acid bacteria in mature Cheddar, Appl. Environ. Microbiol. 65 (1999) 3418–3426.

[14] Fitzsimons N.A., Cogan T.M., Condon S., Beresford T., Spatial and temporal distribution of non-starter lactic acid bacteria in Cheddar cheese, J. Appl. Microbiol. 90 (2001) 600–608.

[15] Giraffa G., Sisto F., Susceptibility to vancomycin of enterococci isolated from dairy products, Lett. Appl. Microbiol. 25 (1997) 335–338.

[16] Grappin R., Beuvier E., Bouton Y., Pochet S., Advances in the biochemistry and microbiology of Swiss-type cheeses, Lait 79 (1999) 3–22.

[17] Husson-Kao C., Mengaud J., Gripon J.C., Benbadis L., Chapot-Chartier M.P., Characterization of *Streptococcus thermophilus* strains that undergo lysis under unfavourable environmental conditions, Int. J. Food Microbiol. 55 (2000) 209–213.

[18] Isolini D., Grand M., Glättli H., Selektivmedien zum Nachweis von obligat und fakultativ heterofermentativen Laktobazillen, Schweiz Milchwirtsch. Forsch. 19 (1990) 57–59.

[19] Jeanson S., Berthier F., Grappin R., Beuvier E., Heat resistance of wild *Lactococcus lactis* strains under a thermal gradient of cooked cheese, in milk and in mini-cheeses, Lait 83 (2003) 379–396.

[20] Lick S., Keller M., Bockelmann W., Hellier K., Rapid identification *of Streptococcus thermophilus* by primer-specific PCR amplification based on its lacZ gene, Syst. Appl. Microbiol. 19 (1996) 74–77.

[21] Terzaghi B.E., Sandine W.E., Improved medium for lactic streptococci and their bacteriophages, Appl. Microbiol. 29 (1975) 807–813.

[22] Thierry A., Salvat-Brunaud D., Madec M.N., Michel F., Maubois J.L., Swiss cheese ripening: dynamics of bacterial populations and evolution of the aqueous phase composition for three industrial cheeses, Lait 78 (1998) 521–542.

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