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Abstract

Bacteria and yeasts are important sensory factors of raw-milk cheeses as they contribute to the sensory richness and diversity of these products. The diversity and succession of yeast populations in three traditional Registered Designation of Origin (R.D.O.) Salers cheeses have been determined by using phenotypic diagnoses and Single-Strand Conformation Polymorphism (SSCP) analysis. Isolates were identified by phenotypic tests and the sequencing of the D1–D2 domains of the 26S rRNA gene. Ninety-two percent of the isolates were identified as the same species in both tests. Yeast-specific primers were designed to amplify the V4 region of the 18S rRNA gene for SSCP analysis. The yeast species most frequently encountered in the three cheeses were *Kluyveromyces lactis*, *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Candida zeylanoides* and *Debaryomyces hansenii*. Detection of less common species, including *Candida parapsilosis*, *Candida silvae*, *Candida intermedia*, *Candida rugosa*, *Saccharomyces unisporus,* and *Pichia guilliermondii* was more efficient with the conventional method. SSCP analysis was accurate and could be used to rapidly assess the proportions and dynamics of the various species during cheese ripening. Each cheese was clearly distinguished by its own microbial community dynamics.
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Keywords: Yeasts; Dynamics; SSCP; 18S rDNA genes; Salers cheese

Introduction

Bacteria and yeasts are an important determinant of the quality of cheeses made from raw milk because they enhance the richness and diversity of the taste of these products. Bacteria alone cannot explain the diversity of the sensorial properties of cheese [8,9]. A comprehensive study on yeast communities in cheeses could help to explain how the sensory qualities of cheeses develop. Several molecular methods are available for microbial identification but only a few have been applied to yeasts isolated from cheeses. These methods include restriction fragment length polymorphism (RLFP) analysis of Intergenic Transcribed Spacers (ITS) [21], randomly amplified polymorphic DNA (RAPD) [1,22], and 26S rRNA gene sequencing with primers NL1 and NL4 [5]. These culture-dependent methods are now being replaced by direct molecular methods without culture. Indeed, Temperature Gradient Gel Electrophoresis (TGGE) has been used to profile wine yeasts [12] and Denaturing Gradient Gel Electrophoresis (PCR–DGGE) has been used to evaluate yeast biodiversity in raw milk [5]. By applying these methods, it is possible to evaluate the dynamics of yeast populations.

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The objective of this study was to evaluate the diversity of yeasts and their dynamics during ripening in three different raw-milk RDO Salers cheese by combining a direct molecular approach relying on the 18S rDNA analysis by Single-Strand Conformation Polymorphism (SSCP) and phenotypic and molecular identification of isolates of the same cheeses. The SSCP methodology used is based on the direct analysis of 18S rRNA gene pool, after PCR amplification and separation in non-denaturing conditions. It provides a rapid fingerprint of a complex microbial community including dead and alive microbial cells. SSCP has been applied to study microbial communities in water [17], in compost of organic agricultural substrate [20], and in anaerobic digestors [16,29]. This technique has also been used in clinical microbiology to rapidly differentiate bacteria from blood culture [26]. SSCP analysis has never been applied to cheese yeast population but it has been used to investigate bacterial dynamics in the same Salers cheeses [4,9].

Materials and methods

Cheeses samples

Cheese samples were obtained from three farms (A, B, and C), in the Salers cheese production area and selected for different sensory features. Salers cheeses were exclusively produced with raw milk stored in a wooden container (the "gerle"). Milk was neither heated nor refrigerated after milking. No starter culture was added. Samples from three cheeses were collected 1 day after cheesemaking and after 8, 30, 90 and 150 days of ripening. Sub-samples were stored at $-80\,^\circ$C until analyzed.

Total DNA isolation from cheese samples

One gram of cheese was mixed with 1 ml of 4 M guanidine thiocyanate–100 mM Tris (pH 7.5) and 125 µl of 10% N-lauroylsarcosine and blended with a reciprocating shaker (MM2000; Kurt Retsch) and frozen at $-80\,^\circ$C. Phenol/chloroform extractions were used to extract total DNA from samples [9]. The DNA solutions were stored at $-20\,^\circ$C.

Primer design

A set of specific primers was developed by comparing gene sequences encoding 18S rRNA (18S rDNA). These sequences, characteristic of most yeast species found in dairy products (26 different species), were aligned by using Multalin version 5.4.1 (multiple sequence alignment with hierarchical clustering) [6]. We designed two conserved primers flanking a variable region in the V4 portion of the 18S rDNA: forward primer Lev1 (5'-AGTTGTTGCAGTTAAAAAG-3') and reverse primer Lev2 (5'-CCTATTAATCATTACGA-3'). Primer specificity was tested as part of the Ribosomal Database Project (RDP) [18] and Blast, and was experimentally tested with a series of 15 reference yeast strains and Salers isolates (Table 1), with *Geotrichum candidum* a positive control and several species of *Lactobacillus*, *Leuconostoc*, *Lactococcus* and *Pediococcus* as negative controls.

PCR–SSCP amplification and SSCP electrophoresis

The primers used to amplify the 270 bp of the 18S rRNA gene were labeled with 5'-hexachloro derivative of fluorescein (HEX: Lev1) and 5'-fluorescein phosphoramidite (NED: Lev2), purchased from Applied Biosystems (Courtaboeuf, France). PCR was performed in a total volume of 25 µl containing $1\times$ Ez buffer (Applied Biosystems), 2.5 mM of Mn $(OAc)_2$ solution (Applied Biosystems), 0.5 µM of each primer, 0.5 mM dNTPs, 2 U of r*Tth* polymerase (Applied Biosystems) and 10 ng of the extracted DNA. The thermocycler was programmed with an initial denaturing step at 94 $^\circ$C for 5 min, followed by 30 cycles of 94 $^\circ$C for 30 s, 47 $^\circ$C for 30 s and 72 $^\circ$C for 60 s, with a final extension at 72 $^\circ$C for 7 min. The amplification products were resolved on a 1% agarose gel and stained with ethidium bromide. PCR products were purified with a StrataPrep^{TM} PCR purification Kit (Stratagene, Saint Quentin en Yvelines, France) following the manufacturer's instructions and then analyzed by SSCP [9].

Cheese 18S rDNA amplification and cloning

The 18S rDNA gene was amplified with unlabeled primers Lev1 and Lev2 from two different cheeses. Each reaction tube contained PfuUltra buffer $1\times$ (Stratagene, Amsterdam, The Netherlands), 0.2 mM of each dNTP, 0.5 µM of each primer, 10 ng of purified DNA and 1.25 U of PfuUltra polymerase (Stratagene) adjusted to a total volume of 25 µl. After initial denaturation at 94 $^\circ$C for 5 min, 30 cycles were performed at 94 $^\circ$C for 30 s, 47 $^\circ$C for 30 s and 72 $^\circ$C for 60 s, followed by a final extension step of 72 $^\circ$C for 7 min. PCR products were ligated in the pCR4Blunt-TOPO vector and transformed into *Escherichia coli* TOPO10 OneShot cells as specified by the manufacturer (Invitrogen, Cergy Pontoise, France).

Identification of SSCP peaks

To assign SSCP pattern peaks to species, the 18S rRNA gene clone libraries were analyzed by SSCP. SSCP–PCR was performed on the clones and the resulting peaks were compared with the SSCP patterns of the cheese. Variable

Table 1. SSCP analysis of 18 S rRNA genes of 15 yeast strains and Salers isolates (three repetitions)

Species	Strains	A[a]	Species	Strains	B[b]
		Elution in scan[c]			Elution in scan
C. catenulata	CBS[d] 565	7246	C. catenulata	CBS 565	7378
Y. lipolytica	CLIB[e] 183	7327	Y. lipolytica	CLIB 183	7450
C. rugosa	CLIB 613	7780	C. parapsilosis	Salers isolate	7948
D. hansenii var hansenii	CLIB 197	7792	D. hansenii var hansenii	CLIB 197	7985
C. intermedia	CBS 572	7796	C.intermedia	CBS 572	7993
C. krusei	CLIB 308	7858	C. krusei	CLIB 308	8078
P. membranifaciens	CLIB 212	7859	P. membranifaciens	CLIB 212	8089
P. fermentans	CLIB 198	7883	C. rugosa	CLIB 613	8117
C. parapsilosis	Salers isolate	7949	P. fermentans	CLIB 198	8125
C. silvae	Salers isolate	8021	C. zeylanoides	CLIB 199	8276
Kl. lactis	CLIB 196	8060	S. dairenensis	CLIB 175	8336
Kl. marxianus	CLIB 282	8093	T. delbrueckii	CLIB 230	8339
S. cerevisiae	CLIB 227	8120	Kl. lactis	CLIB 196	8339
T. delbrueckii	CLIB 230	8129	S. cerevisiae	CLIB 227	8393
S. dairenensis	CLIB 175	8129	S. unisporus	CLIB 234	8402
C. zeylanoides	CLIB 199	8174	Kl. marxianus	CLIB 282	8411
S. unisporus	CLIB 234	8204	C. silvae	Salers isolate	8479

C. = Candida, S. = Saccharomyces, Kl. = Kluyveromyces, T. = Torulaspora, P. = Pichia.
[a]Elution on HEX-labeled forward strand.
[b]Elution on reverse strand NED.
[c]Unit of GeneScan software on the SSCP pattern. Elution values represent the average of three analyses. Standard deviation values always < 5.
[d]Central Bureau vor Schimmelcultures, Neederland.
[e]Collection de Levures d'Intérêt Biotechnologique. Grignon, France.

regions of the 18S rRNA gene from 15 representative clones were sequenced by using dye-terminator cycle sequencing ready reaction kits with *Taq* DNA polymerase and primers Lev1 and Lev2. Yeast reference strains and isolates from cheeses also were analyzed by SSCP to create a species pattern database.

Isolation and identification of isolates

Yeasts of the same three cheeses were counted from fresh sample plates. Yeast colonies were grown on an Oxytetracycline Glucose Agar base (OGA) (Biokar Diagnostics, Beauvais, France) supplemented with oxytetracycline (1 mg/ml). One hundred and two colonies were selected from plates at different times of ripening and purified in Yeast Extract Peptone Glucose (YPG) broth (yeast extract 10 g, bacto-peptone 20 g, glucose 40 g and demineralized water to 1000 ml, pH 5.2). On each plate, colonies were selected based on their aspect diversity. The total of picked colonies per plate was always equal to the root square of total number. All cultures were frozen (−80 °C) before identification.

Yeast isolates were identified by using the morphological, biochemical and physiological characters of Kurtzman and Fell [14] and assigned to a species with the dichotomous key of Barnett et al. [3]. Carbon compound assimilation was determined with the API ID

32C kit (Bio Merieux). All tests were performed in duplicate for non-identified isolates.

Molecular identification was performed by sequencing the 26S rRNA gene. Total DNA was extracted from cultures of each isolate according to the miniprep protocol of Hoffman and Winston [13]. The D1/D2 domain of the 26S rRNA gene was amplified by PCR, using primers NL1 and NL4 as described by Kurtzman and Robnett [15]. The sequencing reactions of the forward and reversed strands were carried out with a dye-terminator cycle sequencing ready reaction kit (Applied Biosystems) with AmpliTaq DNA polymerase FS and the NL1 and NL4 primers, respectively. The reaction products were sequenced with an automated DNA sequencer ABI PRISM 310 (Applied Biosystems) and analyzed with sequencing analysis software (Applied Biosystems). The partial 26S rDNA sequences obtained were compared to the sequences deposited in the GenBank DNA database, using the BLAST algorithm [2]. The percentage of similarity with the DNA sequences deposited was determined.

Results and discussion

Discrimination of yeast species by SSCP analysis

To create a species pattern database, the SSCP patterns of pure cultures (15 culture collection strains

belonging to different species frequently encountered in dairy products [11] were determined. In the same way, SSCP patterns of two strains identified below and originating from microbial analysis of the same Salers cheeses were determined too. 18S rDNA was amplified three times with the two primers Lev1 labeled with HEX and Lev2 labeled with NED. Each strain always produced the same pattern, thus confirming the reproducibility of PCR–SSCP analysis. Differences between the forward and reverse strand of the same strain is due to the fact that SSCP distinguishes DNA fragments according to their sequence on basis of single-strand conformation. Taking both strands into account, nearly all species were easily differentiated by SSCP analysis (Table 1). For example, the HEX-labeled strand from *Torulaspora delbrueckii* and *Saccharomyces dairenensis* had a similar pattern, but these two species could be distinguished by analyzing the NED-labeled strand. A similar result was obtained with *Candida krusei* and *Pichia membranifaciens*. *T. delbrueckii* and *Kluyveromyces lactis* had NED-labeled strands that were similar but could be differentiated by analyzing the HEX-labeled strand. Analysis of both strands generally was necessary to distinguish all 17 species.

Direct fingerprinting of yeast diversity in different Salers cheeses by SSCP–PCR

The reproducibility of the PCR–SSCP approach was evaluated by repeating the DNA analysis of a cheese yeast community two times. The two profiles of the same cheese aligned, with the same variations in each peak area (Fig. 1). The yeast community fingerprints from three different cheeses (A, B and C) were evaluated after 8 and 150 days of ripening (Fig. 2, HEX-labeled strand). The 8-day SSCP patterns differed in the number of peaks per sample. After 150 days of ripening, the fingerprints of the same cheese samples had different SSCP–PCR profiles. Some peaks appeared or disappeared in the course of ripening or differed with respect to their size.

Identification of peaks

To identify the sequences corresponding to peaks in the different patterns, a 260-clone library of 18S rRNA genes from the DNA extracted from cheese A at 150 days (Fig. 2 top right) was constructed and each clone was analyzed by SSCP. All of the clones corresponded to one of the four dominant peaks (a, b, d, e). For each peak, five co-eluting clones were sequenced and identified based on the similarity to 18S rDNA sequences deposited in Genbank. SSCP peak identification also relied on the elution of reference strains and yeast isolates (Table 2). Based on the sequence of the clone
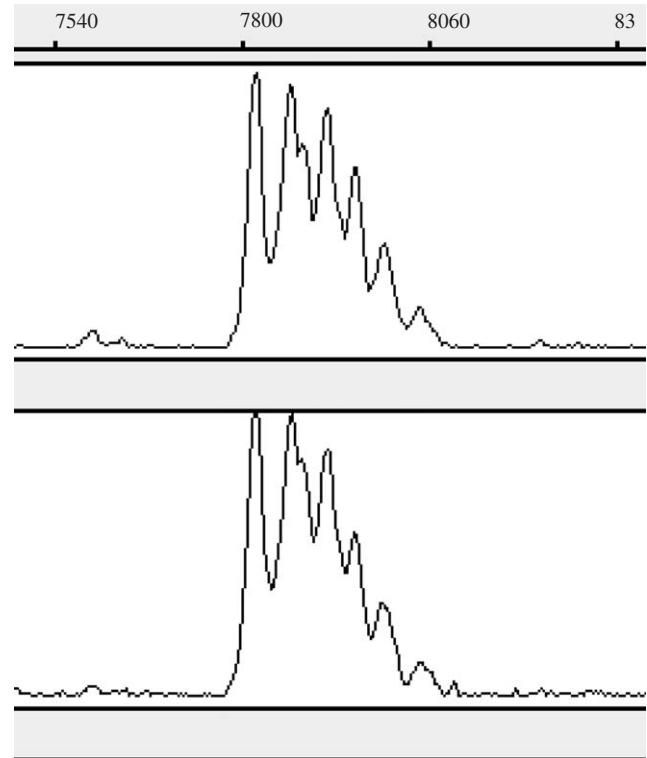

Fig. 1. Reproducibility of PCR–SSCP analysis as assessed by repeating two times the DNA analysis of a cheese yeast community, forward primer labeled with HEX.

18S rRNA gene and co-elution with reference strains and isolates, peak a was *K. lactis*, peak b was *K. marxianus* and peak d was *C. zeylanoides*. Peak e co-eluted with clones identified as *D. hansenii var. hansenii* and *D. hansenii var. fabryii* with a same percentage of similarity with strains in Genbank database. However, peak e had a different pattern to that of the *D. hansenii var. hansenii* type strain. Peaks c and h co-eluted, respectively, with the reference strains for *S. cerevisiae* and *S. unisporus*. Peaks f and g co-eluted with isolates identified as *C. parapsilosis* and *C. silvae*. The other peaks present in patterns (Fig. 2) represent less than 1% of total area or in term of absolute area, less than the value considered as the background and were then considered as non-significant.

Dynamics of yeast populations in cheeses

To analyze the SSCP profiles of cheeses, the peak ratio (the proportion of one peak to the total area of all of the peaks) was calculated in each sample and used as an indicator of changes within the yeast populations. Considering the entire sample times (Table 3), the peaks corresponding to *K. lactis*, *K. marxianus*, *S. cerevisiae*, *D. hansenii* and *C. zeylanoides* were systematically present in the SSCP patterns of all three cheeses. Peaks corresponding to *C. parapsilosis*, *C. silvae* and *S.*
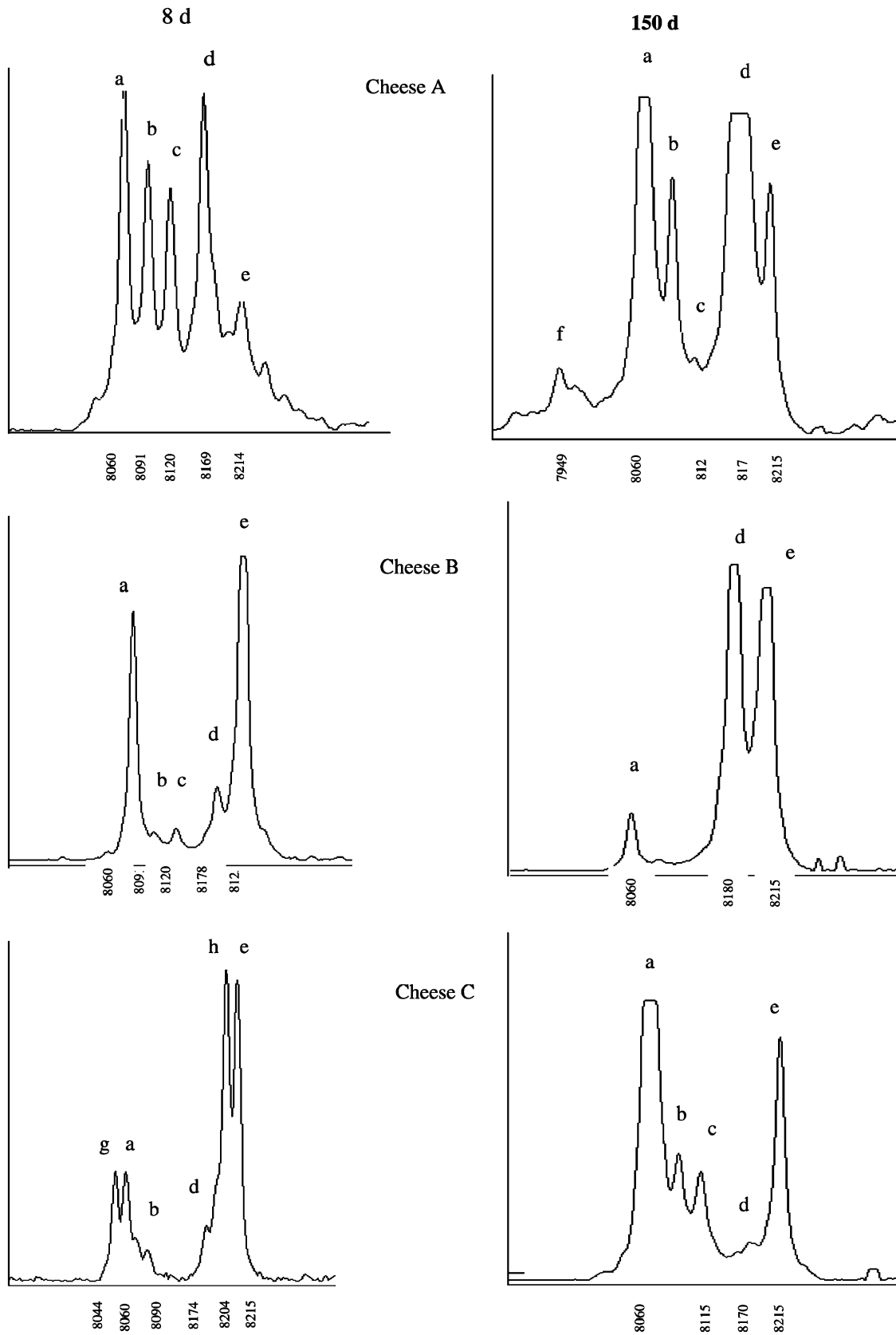
Fig. 2. SSCP patterns of PCR-amplified 18S rRNA gene fragments from yeast communities of three cheeses A, B and C at 8 and 150 d of ripening, forward primer labeled with HEX, Y-axis: fluorescence. X-axis: elution in scans (unit of Genescan software). The position and labeling of peaks discussed in Tables 2 and 3 and in text are indicated.

Table 2. Identification of peaks obtained from SSCP pattern of the yeast community (18S rDNA) by comparison with patterns of reference strains, clones, and isolates

Peaks (Fig. 2)	Reference and isolate SSCP patterns[a]	Assignation according to the elution of: Corresponding clones showing more than 99% identity[b]	GenBank accession no
a	*K. lactis*	K. lactis	CSP508583
b	*K. marxianus*	K. marxianus	AF201299
c	*S. cerevisiae*		
d	*C. zeylanoides*	C. zeylanoides	AB013509.1
e		D. hansenii var. Hansenii	AB106349
		D. hansenii var. fabryii	
f	*C. parapsilosis*		
g	*C. silvae*		
h	*S. unisporus*		

[a]C., Candida, S., Saccharomyces, K., Kluyveromyces.
[b]Percentage of identical nucleotides in the sequence retrieved from the clone and the sequences of the closest relatives found in GeneBank and RDP database.

Table 3. Evolution of the peak ratio of the SSCP patterns of the three cheeses (A, B, and C) at different times of ripening

Peaks	Species	Peak ratios (%)[a]											
		Cheese A				Cheese B				Cheese C			
		Stages of ripening				Stages of ripening				Stages of ripening			
		8[b]	30	90	150	8	30	90	150	8	30	90	150
f	*C. parapsilosis*				4								
g	*C. silvae*	1.8								11			
a	*K. lactis*	23	22	13	30	28	43.4	33	5.0	11	60	66	48
b	*K. marxianus*	20	25	4.2	13	3.8	4.8	4.6		3.6	9.4	12	12
c	*S. cerevisiae*	16	2.8	5.6	3	4.7	9.9	18	0.5		11.5	15	13
d	*C. zeylanoides*	29	30	39	39	12	7.3	17	46	6.2	2.7	0.7	4
h	*S. unisporus*							14		38			
e	*D. hansenii*	11	20	37	12	53	35	9.7	47	30	17	6.9	23
		Total yeast counts (ufc/g)[c]											
		5.53	5.71	5.38	6.68	5.85	5.83	4.95	4.08	6.36	6.20	5.48	4.52

[a]The percent ratio was calculated as the area of one peak to the sum of the area of all peaks of the pattern × 100.
[b]Stage of ripening expressed in days.
[c]Counts on OGA medium + oxytetracyclin.

unisporus were only detected in some cheese at one time. The yeast community of cheese A had a high peak ratio for *C. zeylanoides,* which increased throughout the ripening period and had a peak for *C. parapsilosis*, which was detected only at 150 days. The yeast community of cheese B was dominated by *D. hansenii* after 8 days, but *C. zeylanoides* was dominant at 150 days. In this cheese, *S. unisporus* was not detected before 90 days. The high peak ratios for *C. silvae* and *S. unisporus* after 8 days and their lack of detection later in the ripening period characterized the yeast community of cheese C. During the ripening of this cheese *K. lactis* became dominant.

Dynamics of yeast populations as presented in Table 3 revealed changes in the relative abundance of DNA sequences in the yeast community. Cheese is a closed system and the number of species in the cheese core presumably does not increase throughout the ripening period. Thus, changes in peak ratios are not likely to be due to preferential PCR amplification of some sequences, but rather to changes in the balance of 18S rRNA genes of the species present. The changes of yeast population balance may result from many "events" such as development of one species or lysis of certain cells and degradation of DNA. The total count on OGA medium (Table 3) indicated that the yeast population level

decreased during ripening for two productions. This argues for a lysis of cells but does not provide information on the growth of each yeast species.

Comparison with Salers isolates identification

One hundred and two isolates were selected from the same three cheeses for identification based on phenotypic tests and 26S rRNA sequences. The majority of these partial 26S rRNA sequences were >98% similar to sequences in GenBank. Isolate identification by phenotypic testing and 26S rRNA gene sequencing was congruent for 92% of the isolates. Eight isolates assigned to C. zeylanoides, C. parapsilosis, and K. lactis based on their 26S rDNA sequences were difficult to identify by phenotypic testing because their carbon compound assimilation profiles were not consistent with those of any identified species.

This species inventory, based on isolate identification, was compared with that obtained by the cloning and SSCP methods (Table 4). Both approaches detected K. lactis, K. marxianus, C. zeylanoides, S. cerevisiae and C. parapsilosis. K. lactis and K. marxianus frequently are present in many types of cheese [7,10,23,25,27]. C. zeylanoides often is isolated from Portuguese ewe's cheese [19] and raw milk [5]. S. cerevisiae has been isolated from raw milk [5] and a number of cheeses including Mozarella [7], Gouda [27], Cheddar [28], Camembert [23]. C. parapsilosis was found in Italian raw milk [5].

Table 4. Comparison of presence of different species in three cheeses A, B, and C (all times at once) detected using identification of 94 isolates or SSCP method

Species[a]	Cheese A		Cheese B		Cheese C	
	Isolates	SSCP	Isolates	SSCP	Isolates	SSCP
Kl. lactis	9	d[b]	12	d	9	d
Kl. marxianus	6	d	1	d	7	d
S. cerevisiae	2	d	3	d	7	d
C. zeylanoides	6	d	2	d	2	d
D. hansenii	3	d	3	d		d
C. intermedia	3	nd[c]	2	nd	3	nd
C. silvae		d			3	d
P. guilliermondii			1			
C. parapsilosis	5	d				
C. tropicalis					1	nd
C. rugosa			1	nd	1	nd
S. unisporus			1	d	1	nd
Total	34		26		34	

[a]Identification by 26S DNA sequencing in agreement with phenotypic tests.
[b]Detected.
[c]Non-detected.

The community composition determined by the SSCP method and by isolate identification differed in the detection of D. hansenii, C. silvae and S. unisporus. Neither D. hansenii nor C. silvae were detected by isolate identification but they were detected by SSCP. D. hansenii often is isolated from many types of cheeses. Several isolates were identified as S. unisporus whereas this species was seen in only on a few of the SSCP profiles. This species was found in Armada cheese [25]. We identified isolates of C. intermedia, C. rugosa, C. tropicalis, and P. guilliermondii from RDO Salers cheese by phenotypic identification that were not detected by SSCP. These species have been found sporadically in some dairy products: P. guilliermondii and C. rugosa were found in Italian raw milk [5]. Y. lipolytica previously was isolated from Feta cheese [10], Armada cheese [25], Gouda and Cheddar [27,28] but was not detected in Salers cheese by either method that we used.

The differences in community composition detected by each method reflect the strengths and limitations of each method. Community composition determined by identifying isolates depends on the number of isolates picked from the counting plates and the method used to select those isolates. If the number of isolates selected is too low, then the collection will be not representative of the population. Based on our results, this culture-dependent method is sensitive enough to detect species that are not detected by SSCP analysis. These results are consistent with those of Cocolin et al. [5], who found that more yeast species were detected in raw milk by a culture method than by DGGE. The difficulty in identifying less-common species by direct PCR-based molecular methods (SSCP, T(D)GGE) may be attributed to preferential amplification of dominant populations by PCR and/or to the bias of the SSCP detection system towards the most frequent sequences, i.e. the fluorescent signal of a less common sequence may not be easily distinguished from background noise. This can explain why S. unisporus was only detected after 90 days. At the beginning of ripening, it may be present but subdominant. But the SSCP method is more accurate and rapid to follow dominant population's dynamics.

This study shows the value of combining several methods (identification of isolates and direct molecular method such as SSCP) to describe cheese yeast communities. The use of multiple approaches to characterize populations has not commonly been used to analyze dairy products except by Cocolin et al. [5] who profiled yeast populations in raw milk by using both PCR–DGGE and isolate identification. To our knowledge, this is the first description of the dynamics of yeast communities in cheese by a culture-independent method.

The SSCP method quickly provides an image of the yeast population and permits the visual comparison of several samples. Thus PCR–SSCP is a powerful tool for tracking yeast community composition in fermented

dairy products. Each of the three cheeses had its own community composition profile. These results are consistent with those of Suzzi et al. [24] who also reported that yeast composition could change during the ripening period.

To complete this study, it would be necessary to determine the 18S rRNA SSCP profile, so as to assess the activity of yeasts during ripening. It will then be possible to integrate all these data with those obtained by Duthoit [8] on the bacterial community of this cheese, to better explain their sensorial properties.

Acknowledgements

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