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## Changes in transcription profiles reflect strain contributions to defined cultures of *Lactococcus lactis* subsp. *cremoris* during milk fermentation

Fabien Dachet · Denis Roy · Gisèle LaPointe

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**Abstract** Cheddar cheese production uses mixed starters composed of *Lactococcus lactis* subsp. *cremoris* strains with complementary or competing enzymatic activity. However, strain interactions within the same subspecies are difficult to investigate by conventional microbiological methods. This study uses fluorescent RNA arbitrarily primed PCR (FRAP-PCR) to analyze the association of three *L. lactis* subsp. *cremoris* strains (LL074, LL225, and LL390 with proteinase types: PI, PIII, and PI/PIII, respectively) by monitoring whole transcription profiles. The effect of strain association was demonstrated by distinguishing profiles obtained with pure cultures from those obtained with defined mixed cultures. Both strains LL225 and LL390 dominated culture activity when in dual culture with strain LL074. The three-strain starter was also dominated by LL225 and LL390, in an approximately equal ratio which was stable over 35 generations. Strain LL225 had a stronger inhibitory effect on the growth of strain LL074 than LL390 did, showing incompatibility between strains LL225 and LL074. A new economic and semi-quantitative single-nucleotide polymorphism detection technique was developed and validated the results obtained by FRAP-PCR. Strain disequilibrium detected by FRAP-PCR could be related to inhibition of strain LL074 with a PI-type proteinase by the LL225 strain with a PIII-type proteinase. Strain compatibility could be characterized using these

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methods, leading to an improved understanding of mixed culture association of lactococcal strains.

利用 *Lactococcus lactis* subsp. *cremoris* 不同菌株转录谱的变化判定其对发酵乳的贡献

**摘要** 切达干酪生产使用由具有竞争酶活性或互补酶活性的*Lactococcus lactis* subsp. *cremoris* 菌株组成的混合发酵剂。然而,使用传统的微生物方法很难调查相同亚种中不同菌株间的相互作用。本研究采用荧光RNA任意引物PCR (FRAP-PCR),分析了三个菌株*L. lactis* subsp. *cremoris* (LL074, LL225, LL390, 分别带有蛋白酶型 PI, PIII, PI/PIII) 的联系。菌株间的作用通过区分纯培养和已知的混合培养物来阐明。当LL225和LL390分别与LL074进行两菌株发酵时,LL225和LL390决定培养活力。三菌株的混合发酵剂也是由LL225和LL390控制,稳定性可超过35代。菌株LL225与LL390相比,对菌株LL074的生长具有更强的抑制能力。LL225和LL074显示了不相容性。本文建立了一个新型、经济、半定量SNP(单核苷酸多态性)检测,并通过FRAP-PCR方法对结果进行了验证。由 FRAP-PCR检测的菌株不稳定与LL074菌株(PI型蛋白酶)被LL225 菌株(PIII型蛋白酶)抑制有关。通过这些方法可以确定菌株的兼容性,也增加了对混合培养物中乳球菌株联系的理解。

**Keywords** *Lactococcus* · Mixed culture · Interactions · Cheddar cheese · FRAP-PCR · Transcriptome profiling

**关键词** *Lactococcus* · 混合培养物 · 交互 · 切达干酪 · FRAP-PCR · 转录谱

## 1 Introduction

Multiple strains of *Lactococcus lactis* subsp. *cremoris* are used in combination for cheddar manufacturing, generally to improve final product quality by ensuring proper acidification, proteolysis, bitter peptide removal, and flavor formation. Additional attributes such as bacteriophage resistance and production of inhibitory substances are also considered for the selection of strains (Cleveland et al. 2001). Correct proportions of capsule-forming and ropy strains increase elasticity of the product by releasing optimal concentrations of different exopolysaccharides (EPS) to enhance cheddar rheological properties (Dabour et al. 2006), but many of these EPS-producing strains need to be mixed with strains possessing the capacity to remove bitter peptides (Awad et al. 2005). However, improper microbial activities can lead to acidification problems, rheological defects, or products with atypical flavors caused by overproduction of molecules such as p-cresol, phenylethanol, phenylacetaldehydes, indole, and skatole (Christensen et al. 1999). With respect to the starter culture, strain imbalance can be caused by variations in initial inocula, a shift in pH or temperature condition. Some strains could express genes which result in inhibition of other strains by production of bacteriocins (Picon and Nunez 2007) and also could result in growth stimulation by production of molecules such as folate, which can compensate auxotrophic deficiencies of other strains (Picon and Nunez 2007). Complementation between *L. lactis* subsp. *cremoris* strains in particular is illustrated by improving the production of 3-methylbutanal, as a key flavor compound in a variety of cheese types including cheddar (Smit et al. 2005). One strain provides the amino acids from casein proteolysis while a second strain provides the alpha-keto acid decarboxylase activity.

Defined mixed starters can be difficult to manage when strains of the same species level do not have easily measured phenotypic criteria to separate them. In addition, their nucleotide identity is usually very high (superior to 99%) making genotyping distinction difficult as well. However, recent transcriptomic analyses using arrays have shown that considerable polymorphism exists in gene expression levels between strains of the same subspecies of *L. lactis* subsp. *lactis* in a model cheese (Tan-a-ram et al. 2011). Thus, transcriptome profiling could use gene transcription levels for improving strain differentiation and activity monitoring within the *L. lactis* subsp. *cremoris* subspecies as well. RNA arbitrarily primed PCR (RAP-PCR) has been used to study modifications in bacterial RNA transcription (Wong and McClelland 1994). In this method, the first step is a RNA nonspecific retrotranscription creating a cDNA pool, the next step uses this pool and arbitrary primers to amplify the cDNA population at random positions. The lack of resolution by electrophoresis using acrylamide gels for amplicon visualization has strongly handicapped this technique. Fluorescent RAP-PCR (FRAP-PCR) uses primers marked with a fluorophore allowing capillary electrophoresis to separate amplicons on a sequencer (Crump et al. 2008). These amplicons can also be separated by gel electrophoresis in order to isolate the differentially expressed sequences. The advantages of capillary electrophoresis over other separation processes are the resolution of one nucleotide differences in amplicon size, the low amplicon quantity required (nanogram range) allowing hundreds of runs with the same RNA, and, thus, is a moderately high throughput method. FRAP-PCR was recently adapted for *L. lactis* subsp. *cremoris* in order to determine the effect of milk treatments and conditions on starter culture gene expression (Dachet et al. 2010).

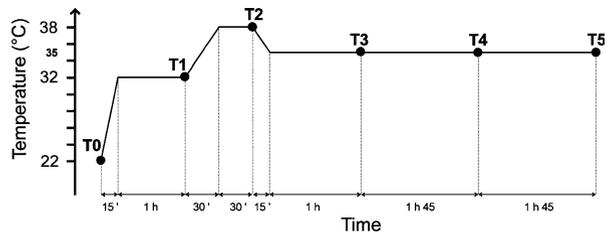
The aim of this study was to use FRAP-PCR to monitor whole transcription profiles of *L. lactis* subsp. *cremoris* in multiple strain cultures during milk fermentation for cheddar cheese manufacture. In order to validate the FRAP-PCR results and to detect the presence of specific strains in a mixed culture, some amplified fragments extracted after acrylamide gel electrophoresis were cloned and sequenced and a FRET-PCR technique (Förster resonance energy transfer PCR) was developed with probes targeting a single-nucleotide polymorphism (SNP) in the *oppC* gene.

## 2 Materials and methods

### 2.1 Strains and culture conditions

The strains used were *L. lactis* subsp. *cremoris* LL074, LL225, and LL390 (DSM Food Specialties, Inc, NJ, USA). UHT milk was used for all cultures to avoid the risk of contamination from bacterial RNA (still present in pasteurized milk (Gunasekera et al. 2002)) and for thermally inactivating RNase. Bacteria were cultured twice, the first preculture of 18 h at 22 °C (Okigbo et al. 1985) was inoculated at 1% (v/v) from frozen glycerol stock and was used to inoculate the second at 1% (v/v). The second preculture was incubated for 16 h at 22 °C and was used as the inoculum at 3% (v/v) for a modified Pearce test at the  $T_0$  time point (Fig. 1). The temperature and time profile of the Pearce test simulates conditions

**Fig. 1** Temperature profile of the modified Pearce test. Samples were taken at T5



used in cheddar cheese making and is used to evaluate the acidification rate of starter cultures (Pearce 1969). In order to differentiate the effect of mixed culture, single strains were cultivated separately to establish profiles without association. To test a short-term association, single strains were cultivated separately then combined at the  $T_0$  time point of the Pearce test. To test longer periods of association, the strains were combined in an equal ratio (inoculum of 0.33% each) then cultivated together over two or five successive mixed precultures before inoculating the Pearce test at  $T_0$ .

## 2.2 DNA extraction

The cell pellet from 7 mL of fermented milk was suspended in 500  $\mu\text{L}$  of lysis buffer (30  $\text{g}\cdot\text{L}^{-1}$  lysozyme in TE consisting of 10  $\text{mmol}\cdot\text{L}^{-1}$  Tris-HCl, 1  $\text{mmol}\cdot\text{L}^{-1}$  EDTA, pH 8) and incubated for 1 h at 37 °C. A volume of 1 mL of phenol/chloroform/isoamyl alcohol (25:24:1) at 46 °C was added along with 100  $\mu\text{L}$  of beta-SDS (10% sodium dodecyl sulfate, 1% beta-mercaptoethanol). After incubation for 20 min at 46 °C, the phases were blended by agitation then separated by centrifugation in a precooled centrifuge (20,000 $\times g$  for 10 min at 4 °C). One milliliter of the aqueous phase was withdrawn, mixed with 1 mL of chloroform, vortexed, and centrifuged (20,000 $\times g$  for 10 min at 4 °C). The aqueous phase was withdrawn, mixed with 5  $\mu\text{L}$  of RNase (Roche, Laval, QC, Canada), and incubated for 30 min at 37 °C. Two extractions were carried out with phenol/chloroform/isoamyl alcohol (25:24:1) and a third with chloroform at room temperature, the centrifugation was carried out at 20,000 $\times g$  for 10 min. DNA was then precipitated from the aqueous phase by adding 1/10 volume of 3  $\text{mol}\cdot\text{L}^{-1}$  sodium acetate (pH 5.2) and one volume of isopropanol followed by centrifugation at 20,000 $\times g$  for 15 min. The pellet was washed with 1 ml of ethanol at 80% and collected by centrifugation at 20,000 $\times g$  for 10 min followed by suspension with TE (pH 8) and quantification by Nanodrop™ 1000 (Thermo Fisher Scientific, Wilmington, USA).

## 2.3 Identification of the cell wall proteinase type

Two signature regions of the *prtP* gene were targeted in order to establish whether the three strains produced different proteinase types. The DNA corresponding to the region in the *prtP* gene covering from amino acid 131 to 177 (subtilisin-like binding region) and from amino acid 747 to 763 (remote sequence) of the mature enzyme was sequenced for each of the three strains. Sequencing used 500 ng of genomic DNA with “Master mix 2.0” from BigDye® Terminator v3.1 (Applied Biosystems, Foster City, USA) using each one of the four *prtP* sequencing primers (Table 1). The nine amino acids 131, 138, 142, 144, 166, 177, 747, 748, and 763

**Table 1** Primers and probes used for FRET-PCR, for *prtP* sequencing as well as primers for RT-qPCR with RNA from *L. lactis* subsp. *cremoris* strains

Primer	Sequence (5'-3')
FRETFor	AATTTCACCTTTGATTGCC
FRETRev	AAATGAAGCGACTAATCCAG
Anchor probe	ATTTAATTTATCATATCCATTTTTAATAGATAAACA (FAM)
Sensor probe	(TAMRA) TTTATCACTCTTGAATGTTGT (p)
<i>prtPSP1</i>	TACAGGCACTATATTATGGC
<i>prtPSP2</i>	GCATCAAGACCACTCAAATC
<i>prtPSP3</i>	ATTCCTGATACCGTTAAGC
<i>prtPSP4</i>	GTTGCCACTAGCATCTTTAA
<i>atpA</i> <sup>a</sup>	GCGGTGAGCTTGTTGAGTTTT CAAGTTTTGCGCCATACCAA
<i>recA</i> <sup>a</sup>	ACAGTCGCTCTTCATGCCGT TGCAATTCACCTTCTTTTTGA
<i>fbxA</i> <sup>b</sup>	TCATTAACGAATTTACGAGTAGCA GTGGTTCTGGTATCCCTGAT
<i>mpA</i> <sup>b</sup>	CGATGTTCTGTCCCTTGATA CGGTTAGTGAAGCAGTTTGA
<i>rmlD</i> <sup>b</sup>	TGGTCATTAACAACGGTCAA TTATATCGTTCGACTGCTTGG
<i>tmRNA</i> <sup>b</sup>	TACACAACTTACGCAATGG GTCTCCACAACAACGTATCT
<i>ppa</i> <sup>b</sup>	TCAATATCAATCAATTCTTCTGCT TACGGTCTTGCAATGCTTAA

Bold underlined letter: nucleotide which causes a correct C-G pairing with DNA of strains LL225 and LL390 but causes a C-T mispairing with DNA of strain LL074

(FAM) FAM fluorophore, (TAMRA) TAMRA fluorophore, (p) added phosphorylation

<sup>a</sup> Calibrator genes used for normalization of target gene expression

<sup>b</sup> Target genes for RT-qPCR

(according to the sequence of the SK11 proteinase (Vos et al. 1989)) separate seven classes of PrtP (Exterkate et al. 1993), from group a containing the reference PIII strain present in *L. lactis* subsp. *cremoris* SK11 to group g containing the PI strain *L. lactis* subsp. *cremoris* HP.

## 2.4 RNA extraction and FRAP-PCR

Seven milliliters of fermented milk withdrawn at T5 of the Pearce test were mixed in equal ratio with RnaProtect® (Qiagen, Mississauga, Ontario, Canada) and treated as described previously for cell harvesting, RNA extraction, reverse transcription, FRAP-PCR reactions, amplicon separation, and statistical analysis (Dachet et al. 2010). Briefly, the cell pellet was suspended in 500 µL of lysis buffer (100 g.L<sup>-1</sup> lysozyme, 10% sucrose, pH 5) and incubated for 5 min at 46 °C. One milliliter of Trizol®

(Invitrogen) at 46 °C was added with 200  $\mu\text{L}$  of chloroform and 100  $\mu\text{L}$  of beta-SDS (10% sodium dodecyl sulfate; 1% beta-mercaptoethanol). After incubation for five minutes at 46 °C, the phases were separated by centrifugation in a precooled centrifuge (20,000 $\times g$  for 5 min at 4 °C). One milliliter of the aqueous phase was withdrawn then mixed with 500  $\mu\text{L}$  of isopropanol at room temperature for purification on an RNeasy<sup>®</sup>-column (Qiagen). The manufacturer's washing protocol was followed, except the DNase treatment that was applied on column with 33 units of Superase-In<sup>™</sup> (Ambion, Applied Biosystems, Foster City, USA).

RNA was used at a concentration of 15  $\text{ng}\cdot\mu\text{L}^{-1}$  with 19 units of Superase-In<sup>™</sup> and 2.5  $\mu\text{mol}\cdot\text{L}^{-1}$  of an equal ratio of retrotranscription primers (ST1 to ST13) (Dachet et al. 2010). The reaction was incubated at 65 °C for 5 min, then immediately placed on ice for 1 min and used in the retrotranscription reaction. The final volume of the retrotranscription reaction was 19  $\mu\text{L}$  and contained 2 mM of total dNTPs, 190 units of SuperScript<sup>™</sup> III (Invitrogen, Burlington, Ontario, Canada), 3.7  $\mu\text{L}$  of 5 $\times$  buffer, 5  $\text{mmol}\cdot\text{L}^{-1}$  of dithiothreitol. The mix was first incubated at 25 °C for 10 min then 45 °C for 2 h. The reverse transcriptase inactivation step was applied at 70 °C for 15 min.

A quantity of 20 ng of cDNA was used as template for FRAP-PCR amplification. For 20  $\mu\text{L}$  of final volume, each reaction mixture contained: 1.67  $\text{mmol}\cdot\text{L}^{-1}$   $\text{MgSO}_4$ , 700  $\mu\text{mol}\cdot\text{L}^{-1}$  of total dNTPs, 1.34 units of hot-start Kod polymerase (EMD Bioscience, Novagen<sup>®</sup>, Madison, Wisconsin USA), 2  $\mu\text{mol}\cdot\text{L}^{-1}$  of one nonlabeled primer and 2  $\mu\text{mol}\cdot\text{L}^{-1}$  of one fluorescent primer. The first steps were: 95 °C for 10 min, 35 °C for 40 min and 72 °C for 5 min. Then the following 30 PCR cycles were performed with high stringency cycling: 95 °C, 30 s; 55 °C, 40 s; 72 °C, 60 s. The PCR amplification was mixed with 500  $\mu\text{L}$  of TE (10  $\text{mmol}\cdot\text{L}^{-1}$  Tris, 1  $\text{mmol}\cdot\text{L}^{-1}$  EDTA, pH 8) and loaded on a Microcon<sup>®</sup> YM100 (Millipore, Billerica, Massachusetts, USA). The amplicons were washed four times with successive passages of 500  $\mu\text{L}$  TE on the same column. Elution was carried out with 50  $\mu\text{L}$  of TE, and PCR products were quantified by spectrofluorescence at 260 nm.

In order to distinguish between the real effects of association and the effect of mixing RNA only (amplification bias), two control profiles were created for each replicate with no association among the three strains. First, the single-strain mixed RNA was formed by combining equal ratios of RNA extracted from each of the three separately cultivated strains, using the mixed RNA as template for FRAP-PCR. Second, the single-strain average profile was formed using Excel by calculating the average height of each peak from the profiles of the three single-strain cultures.

## 2.5 Native polyacrylamide gel electrophoresis and amplicon cloning

A quantity of 3  $\mu\text{g}$  of single-strain purified amplicons was loaded on a 6% acrylamide gel (acrylamide/bisacrylamide in a 29:1 ratio). Electrophoresis was run at 130 volts for 20 h at 4 °C. Ethidium bromide was used to stain DNA and the gel was immersed in hot water at 45 °C for 5 min to eliminate background noise. Extraction of the selected differentially expressed bands was done under UV light with a scalpel. The acrylamide bands were fixed in melted 0.6% agarose, electrophoresed at 100 V for 10 min then extracted bands were purified with Qiaex II (Qiagen). Purified bands from profiles of single-strain cultures were further amplified with the

same primer pairs used in the first amplification (either with STF2 and ST12 or STF5 and ST9). PCR was carried out for 30 cycles with ThermoPol polymerase (NEB, Pickering, Ontario, Canada) following the conditions recommended by the manufacturer, under the following cycling parameters: 95 °C, 30 s; 60 °C, 30 s; 72 °C, 1 min with a 90 min final incubation time. Amplicons were purified with Microcon<sup>®</sup> YM-100 (Millipore, Billerica, Massachusetts, USA) and ligated to plasmid pGEM<sup>®</sup> T (Promega, Madison, WI, USA). *Escherichia coli* strain JM109 was transformed with the ligated plasmid and cultured at 37 °C on LB plates containing 80 mg.L<sup>-1</sup> Xgal, 0.5 mmol.L<sup>-1</sup> IPTG and 100 mg.L<sup>-1</sup> ampicillin. Plasmid DNA of selected clones was extracted by alkaline lysis then the insert was sequenced using BigDye<sup>®</sup> Terminator v3.1 (Applied Biosystems). In a total reaction of 10 µL, a quantity of 240 ng of extracted plasmid was added with primer SP6 (5' ATTTAGGTGACACTATAG) at a final concentration of 150 nmol.L<sup>-1</sup>, 2 µL of 5× buffer and 0.5 µL of big dye ready mix. The cycle conditions were 96 °C, 10 s; 50 °C, 5 s; 60 °C, 4 min; and 99 cycles.

## 2.6 Reverse transcription quantitative PCR

In order to confirm differential gene expression of genes found in the extracted acrylamide bands, reverse transcription quantitative PCR (RT-qPCR) measurements were done with 15 ng of cDNA from global retrotranscription of RNA using 13 FRAP-PCR primers (ST1-ST13) (Dachet et al. 2010), on a 7500 real-time PCR system (Applied Biosystems) using the recommended SYBR Green mix (Applied Biosystems) in a final volume of 25 µL. Amplification efficiency was determined with DART-PCR (Peirson et al. 2003), relative gene expression normalized on the average expression of the three strains was assessed with qBase+ (Hellemans et al. 2007) and the reference genes were determined with geNorm (Vandesompele et al. 2002). The primers (Table 1) were designed using PerlPrimer software (Marshall 2004) and were synthesized by IDT (Integrated DNA Technologies, Inc., Coralville, IA, USA). Significance among differentially expressed RNA ratios was determined at the 95% confidence level using the Tukey's test carried out by the "R" statistical package (Development Core Team et al. 2008).

## 2.7 Strain differentiation by FRET probe dissociation curve analysis

Fluorescent adjacent probes for FRET analysis (Föster Resonance Energy Transfer) used a FAM fluorophore (absorption=494 nm and emission=518 nm) at the 3' end of the anchor (probe reporter) and a TAMRA fluorophore (absorption=565 nm and emission=580 nm) at the 5' end of the sensor (probe quencher). The mix used was: forward and reverse primers at 300 nmol.L<sup>-1</sup> each (Table 1), anchor probe at 80 nmol.L<sup>-1</sup> and sensor probe at 200 nmol.L<sup>-1</sup>; 2 U of Thermopol<sup>®</sup> DNA polymerase (NEB), 800 µmol.L<sup>-1</sup> total dNTPs with 20 ng of DNA template in a final volume of 50 µL.

The sensor probe was designed on the strand which maximized the shift in  $T_m$  by a C to T mispriming and by incorporating the SNP in the middle of its sequence. The anchor was designed on a conserved sequence separated by two nucleotides from the sensor. By incorporating a SNP in the sensor, the mismatched nucleotide is detectable by a decrease in the sensor  $T_m$ . This SNP was used to separate strain

LL074 from strains LL225 and LL390. PCR cycles of the SNP assay were: 95 °C, 15 s; 61 °C, 60 s; and 45 cycles followed by a dissociation stage from 35 to 95 °C at 0.036 °C.s<sup>-1</sup>.

On the Applied Biosystems 7500 using SDS 1.2 software, melting curves are usually employed to monitor fluorescence decrease from SYBR Green® and detect  $T_m$  using the inflection point from the derivative curve. In contrast, the adjacent fluorescent probe assay with a TaqMan® simulated detector (FAM reporter and TAMRA quencher) results in an increase of FAM fluorescence from the anchor when the sensor is thermally separated from its template (no more quenching from TAMRA by the FRET effect). In this case, to obtain positive peaks, the derivative curve was transformed by Excel® using invert and opposite. The primers and fluorescent probes were designed using BioEdit (Hall 1999) with the PerlPrimer software (Marshall 2004) to detect auto and hetero hybridization and were synthesized by IDT (Integrated DNA Technologies, Inc.).

### 3 Results

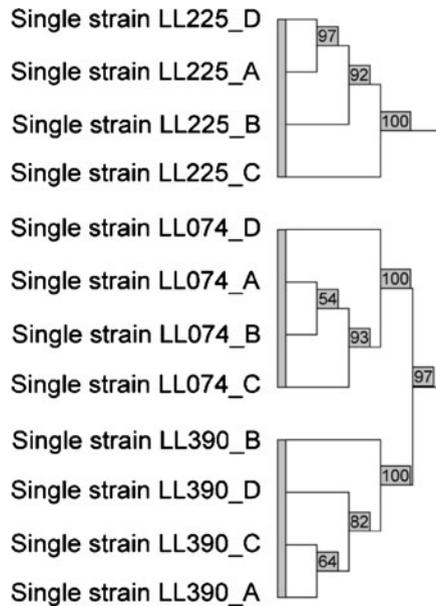
#### 3.1 Identification of the cell wall proteinase type for the three strains

By comparison with the seven classes of cell envelope proteinases (Exterkate et al. 1993), the predicted protein product of the sequenced *prtP* gene regions from strain LL074 has the nine amino acids T, T, A, L, D, I, L, T, H, which correspond to the PI-type proteinase. This proteinase was very similar to the PI sequence from the reference strain *L. lactis* subsp. *cremoris* HP: T, T, D, L, D, I, L, T, H (except for one difference in position 142). Strain LL225 has the nine amino acids T, T, A, L, D, L, R, K, N characteristic of the proteinase from the PIII-type group b present in *L. lactis* subsp. *cremoris* strain AM2. Strain LL390 has two types of proteinase: a PI type (T, T, S, L, D, L, L, T, N) and a PIII type (T, T, A, V, N, L, R, K, N). The predicted sequence of the PI-type proteinase was identical to that of the PI variant of group e present in *L. lactis* subsp. *cremoris* strain Wg2. The PIII-type proteinase sequence is similar to that of the PIII reference group present in *L. lactis* subsp. *cremoris* strain SK11: S, K, A, V, N, L, R, K, N (except for two differences in positions 131 and 138) and is also most similar to the PIII variant represented by *L. lactis* subsp. *cremoris* AM2: T, T, A, L, D, L, R, K, N (except two differences in positions 144 and 166).

#### 3.2 Differential expression profiling of pure strain cultures by FRAP-PCR

Hierarchical clustering analysis showed that independent replicate experiments using the same strain in pure culture were grouped together at a high bootstrap value of 100% and that these replications were separated by strain (Fig. 2). A total of 14 bands differentially overexpressed in one strain were identified by comparing profiles from pure strain cultures by polyacrylamide gel electrophoresis of FRAP-PCR amplimers from individual primer pair amplifications (Fig. 3). Visually on gel, five of these bands were specifically overexpressed by strain LL074, while four bands were specifically overexpressed by strain LL225 and five bands were specifically overexpressed by strain LL390. Sequencing of 12 clones

**Fig. 2** Hierarchical clustering of electropherograms from single-strain cultures at T5 of the modified Pearce test. Clustering was carried out using average linkage and the Euclidian distance metric with 10,000 permutations. *A–D* The four independent experimental repetitions

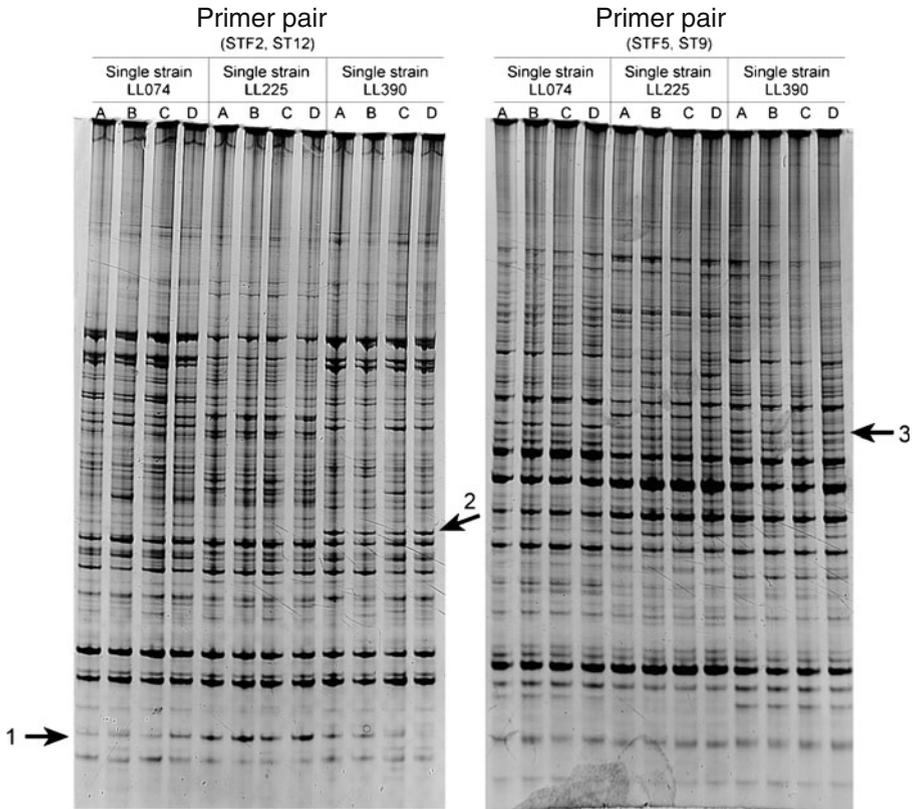


per band extraction showed that each band contained a number of different sequences that comigrated in the same band. The number of different sequences ranged from a pure amplimer per band to a mix of seven discrete amplimers per band (Table 2).

For bands 1 to 5, differentially overexpressed for strain LL074, no clones were obtained in order to validate expression by RT-qPCR. Band 4 contained a sequence which had a single-nucleotide polymorphism specific to strain LL074. This SNP (guanine for strain LL225 and LL390, thymine for strain LL074) was located in the primer binding site of the fluorescent arbitrary primer STF5, thus facilitating hybridization to its target within the *oppC* gene. From the three different sequences in band 2, only the RNA of dehydrorhamnose reductase was found to be significantly underexpressed in LL225 (Table 3). Bands 6, 7, and 9 contained sequences of fructose biphosphate aldolase, the transposase for IS1216, and tmRNA respectively, which were found differentially overexpressed in strain LL225. Band 11 contained the sequence of an inorganic pyrophosphatase which had a significantly higher expression in strain LL390.

### 3.3 Effect of co-culture on global transcriptome profiles

Principal component analysis (PCA) shows that the sum of the variation explained by the two first eigenvectors is more than 50% of the total variance of the FRAP-PCR data (Fig. 4a). The four clusters of the actual cultures are well separated from the two clusters of control profiles. The profiles of the single strains LL225 and LL390 have opposite loadings on the first component, indicating a major overexpression or underexpression of particular distinctive peaks for each of these two strains. The profile of strain LL074 was equidistant from those of strains



**Fig. 3** Native polyacrylamide gel electrophoresis of RAP-PCR amplimers from RNA extracted from single-strain cultures. Differentially expressed bands obtained using primer pairs STF2 with ST12 and STF5 with ST9 were extracted from gels. *A–D* The four independent replicate profiles. *1*, Band containing the fructose biphosphate-aldolase sequence; *2*, band containing the inorganic pyrophosphatase sequence; *3*, band containing the dehydrohamnose reductase sequence

LL225 and LL390. The single-strain average profile clusters with the single-strain mixed RNA profiles, located at the intersection of the two components. The central location of the mixed RNA and average profiles indicates that there is no variation in peaks along these components. The large cluster size of the single-strain mixed RNA compared with single-strain average profiles could be caused by errors in RNA spectrophotometry measurement between the four different replicates, causing wider variation in peak sizes. All other clusters contribute particular peak variations not present in the central cluster.

#### 3.4 Effect on transcriptome profiles of the number of successive mixed cultures

A total of 38 peaks were detected as significantly differentially expressed in mixed strain profiles compared with single-strain profiles. PCA shows that the grouping of mixed strain cultures differs from the single-strain cultures (Fig. 4b). The first component explains more than 50% of the variance, indicating major significance of this axis (Ramette 2007) with only minor influence of the

**Table 2** Amplimers contained in bands from acrylamide gels that were extracted, cloned, sequenced, and expression quantified by RT-qPCR

Strain	Band number	RT-qPCR confirmation of differential expression	Number of different sequences identified <sup>a</sup>
LL074	1	0	2
	2	0	3
	3	0	6
	4	0	4
	5	0	6
LL225	6	1	1
	7	1	7
	8	0	5
	9	1	2
LL390	10	0	1
	11	1	3
	12	0	5
	13	0	2
	14	0	3

<sup>a</sup> From a total of 12 clones analyzed for each band

second component. The three clusters with multiple subcultures can be distinguished from the single-strain mixed RNA control profiles on the first component. In addition, differences can be observed along the second component between the groups with short term association (located in the center) compared with two or five successive mixed cultures representing longer periods of association. As there appears to be greater spread indicating higher variability among replicates using five subcultures, two successive mixed subcultures were selected for subsequent experiments.

### 3.5 Strain equilibrium during milk acidification

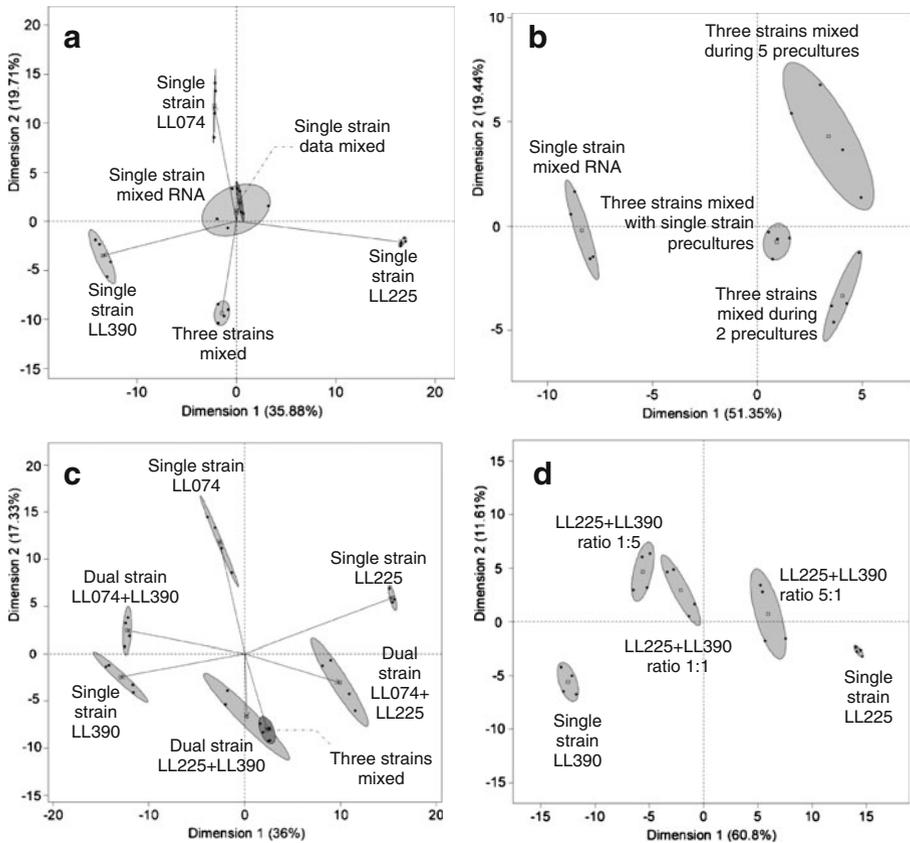
To determine the final strain composition of the mixed culture, three different dual strain mixed cultures were carried out using LL074 with LL225, LL225 with LL390,

**Table 3** Normalized expression of genes which were selected as significantly differentially expressed by FRAP-PCR

Expression level of gene target <sup>a</sup>					
Strain	<i>fbxA</i> fructose biphosphate aldolase	<i>tnpA</i> transposase of IS1216	<i>rmID</i> dehydrorhamnose reductase	tmRNA	<i>ppa</i> inorganic pyrophosphatase
LL074	0.42±0.06	0.05±0.02	3.86±0.56	0.03±0.03	0.70±0.19
LL225	3.36±1.20*	30.15±12.48*	0.07±0.01*	76.27±39.19*	0.74±0.26
LL390	0.76±0.25	1.29±1.59	3.93±0.84	1.58±1.99	2.11±0.38*

<sup>a</sup> Gene expression was scaled to the average expression of the three strains and compared using the Tukey's test at 95% confidence

\* $P < 0.05$ , indicates significantly different values



**Fig. 4** Principal component analysis of FRAP-PCR profiles obtained from T5 of the Pearce test carried out with **a** three independently cultured single strains compared with the single-strain mixed RNA and the three strain mixed culture. **b** Pure or mixed cultures after zero, two, or five successive cultures. **c** Single-strain cultures, three dual strain cultures, and the three strain-mixed culture. **d** Cultures using three ratios of strains LL225 and LL390 compared with single-strain cultures. *Gray shading* encloses replicate experiments of the same type with the group median (*squares*) as the cluster center and terminus of the vector

and LL074 with LL390 in equal inoculation ratios. At the T5 point of the Pearce test, the profiles of the three-strain mixed cultures were similar to the dual strain mixed culture of LL225+LL390 (Fig. 4c). This suggests that strain LL074 could have been washed out from the three-strain mixed culture, or that the strain does not contribute any distinctive RNA peaks. There is very little separation of the dual strain LL074+LL390 cluster from the single-strain LL390 cluster. This close profile similarity indicates only slight impact of strain LL074 on the overall dual culture profile with strain LL390. The separate location of the LL074+LL225 profile indicates peak differences for this dual strain culture. The SAM algorithm (FDR=0.4%, 10,000 permutations) was used to discard from the LL074+LL225 profile the peaks also found in the profile of the LL225+LL390 culture, in order to remove common peaks associated with strain LL225. PCA of the restricted data set could not distinguish the profile of single-strain LL225 from the dual strain LL074+LL225 profile. This also suggests that strain LL074 does not contribute distinctive peaks to the profile of the interactive culture.

### 3.6 Effect of strain imbalance on mixed culture transcriptome profiles

As the three strain mixed culture profile appeared to be composed of RNA from strains LL225 and LL390, the ratio of these two strains was modified in order to determine how this affected the profile of the interaction. Strains LL225 and LL390 were thus cultivated at a ratio of five to one as well as one to five. The profiles of the two imbalanced dual strain cultures were each skewed towards the cluster representing the single strain which was in excess, suggesting no significant dominance of strain LL225 or LL390 in the dual strain culture and so a possible stable strain ratio of around 1:1 (Fig. 4d).

### 3.7 Differentiation of strain LL074 from strains LL225 and LL390 using fluorescent adjacent probes

No overexpressed peak could be identified from profile comparisons in order to identify strain LL074 by RT-qPCR. However, strain LL074 has one polymorphism (G to T) in the *oppC* gene that favored hybridization with the fluorescent primer STF5. This SNP was used to design a fluorescent adjacent probe in order to distinguish LL074. Using DNA from single-strain LL074, the sensor  $T_m$  was 43.5 °C (Fig. 5a) while the sensor  $T_m$  was 52.5 °C using DNA from strains LL225 or LL390 (Fig. 5b, c). The amplified DNA from both the three strain culture and the dual strain culture of LL074+LL225 shows a sensor  $T_m$  of 52.5 °C (Fig. 5d) with no peak at 43.5 °C characteristic of strain LL074, indicating the disappearance of strain LL074 below the detection level of the test. The detection limit was determined to be 1 in 20 by analyzing mixtures of the two strains where strain LL074 was gradually reduced to a ratio of 1:20. The dual strain LL074+LL390 culture has two peaks (Fig. 5e). The peak from single-strain cultures of LL074 and LL390 mixed in a ratio of 1:5 (17% of DNA from strain LL074 and 83% of DNA from strain LL390) showed the same height as the dual strain culture LL074+LL390 inoculated at an initial ratio of 1:1 (Fig. 5f). This indicates that strain LL074 was not removed from this dual strain culture, but there was a shift in proportion of the two strains, explaining the predominance of strain LL390 over strain LL074 that was also observed with FRAP-PCR.

## 4 Discussion

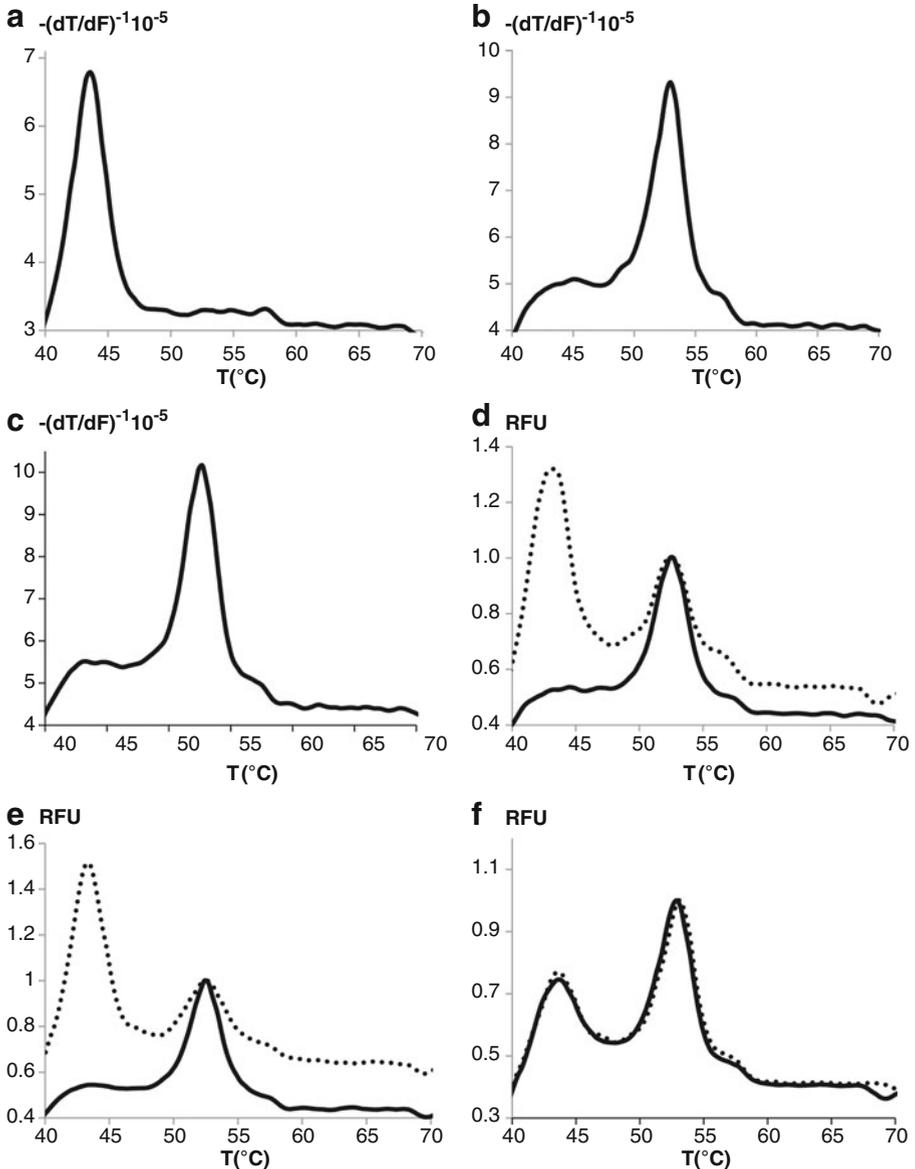
This is the first report of the application of FRAP-PCR to study a lactococcal association in defined mixed culture simulating milk fermentation during cheddar manufacturing. The effect of strain association was demonstrated by distinguishing whole transcriptome profiles obtained with pure cultures from those obtained with defined mixed cultures. FRAP-PCR is not handicapped by problems of irreproducibility inherent to arbitrary PCR (Jurecic et al. 1998; Zhao et al. 1995), and does not show technical bias due to competition during PCR. Previous false positive problems could have been caused principally by the use of acrylamide electrophoresis, as many different amplimers (differentially expressed and constitutive) comigrate in the same band on gel (Bauer et al. 1994). This was shown by clone analysis, leading to identification of three genes (*fbmA*, *tnpA*, and

**Fig. 5** Fluorescent adjacent probe detection using DNA extracted from milk culture of *A* single-strain culture of LL074 (sensor  $T_m=43.5$  °C). **b** Single-strain culture of LL225 (Sensor  $T_m=52.5$  °C). **c** Single-strain culture of LL390 (Sensor  $T_m=52.5$  °C). **d** Fluorescent adjacent probe detection in relative fluorescence units standardized on the LL225 peak showing comparison between the three strain culture of LL074+LL225+LL390 in a 1:1:1 ratio (*solid line*) and a mix (1:1:1) of DNA from the three strains cultivated separately (*dotted line*). **e** Comparison between the dual strain culture of LL074+LL225 in a ratio of 1:1 withdrawn at T5 of the modified Pearce test (*solid line*) and a mix of DNA from strains cultivated separately then mixed at a ratio of 1:1 (*dotted line*) (sensor  $T_m=43.5$  and  $52.5$  °C). **f** Comparison between DNA from a two strain mixed culture of LL074+LL390 with an initial ratio of 1:1 withdrawn at T5 of the Pearce test (sensor  $T_m=43.5$  and  $52.5$  °C) and a mix of DNA from single-strain cultures of LL074 and LL390 cultivated separately and mixed in ratio 1:5 (*dotted line*) (sensor  $T_m=43.5$  and  $52.5$  °C)

tmRNA) that could be used to reveal the transcriptional activity of strain LL225 and one gene (*ppa*) differentially expressed by strain LL390. The higher expression of *fbA* in strain LL225 could lead to a relatively elevated rate of glycolysis compared to the other two strains at the end of the Pearce test. The lower expression of *tnpA* in strains LL074 and LL390 compared to LL225 may be due to the integration site of this IS6-family insertion element, or to differences in promoter function. In strain SK11, 11 complete IS6 elements were found in the sequence of four plasmids (Siezen et al. 2005), so plasmid location of these genes may lead to higher expression levels in strain LL225 due to copy number. FRAP-PCR can, thus, be applied as an alternative to genome sequencing or to SSH for identifying biomarkers useful for distinguishing strains during mixed culture (Ndoye et al. 2011).

Incubating the three strains together or mixing RNA of independently cultured strains gave transcriptome profiles that were not clustered together. Consequently, mixing of strains resulted in activation and repression of particular peaks thus indicating some interactions between strains during the acidification of milk. This difference in profile was apparent at the first mixed culture, and continued over 35 generations (strains grown for five successive mixed cultures). The greater spread in profile positions observed as the number of successive mixed cultures increased indicates that, for this strain combination, the duration of the mixed culture of strains should be controlled to minimize variation (Stadhouders and Leenders 1984).

To our knowledge, this is also the first report of the use of this type of FRET adjacent probes on an Applied Biosystems real-time PCR system as these two technologies were reputed to be incompatible (Marras 2006). Compared with Taqman<sup>®</sup> assays, SNPs can be detected at a reduced cost by using only one fluorophore per probe, a conventional DNA polymerase, and a simple TAMRA fluorophore as quencher. By combining information on transcriptomes from FRAP-PCR with strain detection by FRET-SNP, the effect of mixed culture on strain equilibrium could be demonstrated. In the presence of strain LL225, RNA, and DNA of strain LL074 was below the detection level (determined to be 1:20). Growth of strain LL074 was more inhibited by strain LL225 than by LL390 as DNA from strain LL074 was still detected at a concentration of 1:5 in the LL074:LL390 dual strain culture. This wash-out could be explained by the release of PIII-type peptides by strain LL225, which could inhibit the growth of the PI-type strain LL074 (Flambard et al. 1997). Other causes of wash-out could be the sensitivity of strain LL074 to bacteriophage possibly released by strain LL225 (LL225 and LL390 could



be resistant to the bacteriophage and LL074 sensitive) or by release of bacteriocins (Picon and Nunez 2007). However, inhibition by antimicrobial activity was not observed among these strains. Strain incompatibility could be due to numerous factors, and they may not respond to stress in the same fashion. Such interactions lead to variations in starter culture composition which can have an ultimate impact on starter performance and consequently on product quality. Early diagnosis of strain incompatibility could thus facilitate the selection of high performance starters and the maintenance of strains with desired properties within those mixed starters.

When strain LL074 was mixed with strain LL225, the resulting transcriptome profile was distinguishable from that of both single-strain profiles, indicating the presence of new peaks due to an interactive response. This also occurred for the LL225+LL390 mixed culture. By comparing the pure and mixed culture profiles, specific peaks present only in the associations can be segregated. The residual profile of dual strain LL074+LL225 is not different from the LL225 single-strain culture after subtraction of the equivalent association peaks that were found in the profile of the dual strain LL225+LL390. This indicates that the difference between the dual strain LL074+LL225 culture and the single-strain LL225 was due to the association-specific peaks also distinctive of the dual strain LL225+LL390 profile. As strain LL390 was not present in the LL074+LL225 mix, the separation of cluster LL074+LL225 from cluster LL225 was only due to peak modulation of strain LL225, which was identical when mixed with either strain LL074 or strain LL390. This shows that gene transcription of one strain can be modulated in response to the presence of other strains of the same subspecies. Using a luciferase reporter in a miniature Gouda cheese system, casein proteolysis by a protease positive starter culture was shown to downregulate *dppA* promoter function in *L. lactis* NZ5500, which is a lactose positive but proteolytic negative variant of strain MG1363 harboring an R-IVET clone library (Bachmann et al. 2010). As an example of interspecies interaction, *L. lactis* subsp. *lactis* biovar *diacetylactis* showed a remarkable change in gene expression profile in response to mixed culture with *Staphylococcus aureus*, which was attributed to a decreased growth rate due to glucose competition (Nouaille et al. 2009). In return, the same *L. lactis* strain did not greatly affect the expression of *S. aureus* metabolic genes during mixed culture, but the expression of certain enterotoxin genes was repressed (Even et al. 2009). The use of microarray technology thus reveals the exact gene differences, but requires some knowledge of the genome sequence of both strains. In addition, it is more difficult to apply to screening large numbers of samples for the moment.

The dominance of the transcriptome of strain LL390 over strain LL074 observed by FRAP-PCR was correlated with a shift in cell proportion as the DNA from strain LL390 quantitatively dominates DNA from strain LL074. The partial inhibition of strain LL074 by strain LL390 could be explained by the fact that the strain LL390 was not a pure PIII-type strain. This means that the proportion of PIII-type peptides was probably lower than that of strain LL225, which could lead to an inferior inhibition of the PI-type strain LL074, allowing the presence of a small amount of this strain. The lack of inhibition of strain LL390 by strain LL225 could be explained by the presence of a *prtP* gene coding for a PIII-type proteinase, which should be compatible with other PIII-type strains.

In conclusion, application of FRAP-PCR allowed successful monitoring of a strain association during milk fermentation. This technique easily separated three strains of *L. lactis* subsp. *cremoris* (LL074, LL225, and LL390) from one another and revealed the effects of strain mixing during the temperature profile of cheddar cheese making. Imbalance between these strains could be detected by FRAP-PCR and by using a FRET-SNP technique which showed that the RNA transcriptome profile was correlated with the presence of bacterial strains. Interactions between these three strains could be explained by proteinase type incompatibility resulting in an inhibition of PI-type strains by PIII-type proteinase activity.

As a perspective, the microbial dynamics of mixed strain association during cheese ripening could be characterized by molecular techniques. The evaluation of strain compatibility will prevent inappropriate strain mixing and will help to discover new stable strain associations by eliminating incompatible mixes, thus reducing the effort required to characterize starter cultures. Molecular techniques such as FRAP-PCR could be used to validate proper strain proportions, the stability of existing starter strain combinations and to create new starters by mixing compatible strains. Improved understanding of microbial association will allow a reliable control of new starter composition leading to superior cheddar quality with optimal sensory properties.

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