Dairy Sci. Technol. 90 (2010) 399–412
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DOI: 10.1051/dst/2010020

Available online at:
www.dairy-journal.org

Original article

Transcriptome profiling of lactococcal mixed culture activity in milk by fluorescent RNA arbitrarily primed-PCR

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Received 18 July 2009 – Revised 4 January 2010 – Accepted 28 January 2010
Published online 30 March 2010

Abstract – Thermal treatment of milk is widely used to reduce milk contamination, while CO_2 can be used to prevent bacterial growth and maintain milk quality during storage. These treatments applied before or during cheese manufacture could alter the metabolic activity of starter cultures. Changes in gene expression can be evaluated by differential display methods, so that effects on bacterial metabolic activity can be estimated by variation in transcription profiles. The aim of this study was to develop fluorescent RNA arbitrarily primed-polymerase chain reaction (RAP-PCR) as a method to evaluate the influence of milk CO_2 acidification as well as rennet and salt concentrations on starter gene expression. Comparison with reference conditions showed that gene transcription was influenced according to the extent of thermal treatment as well as by CO_2 acidification followed by different neutralization procedures. Thus, simple acid neutralization after CO_2 acidification was not sufficient to regain the reference transcriptome profile. Starter gene transcription profiles showed important modifications following an increase in NaCl concentration or a decrease in rennet activity from standard conditions used in Cheddar cheese making. Increasing rennet activity results in small changes in the starter RNA profile. Fluorescent RAP-PCR is a promising method for obtaining a better understanding of gene expression profiles of mixed cultures during cheese making.

***Lactococcus* / starter culture / dairy product / carbon dioxide / RAP-PCR / transcription profile**

摘要 – 荧光 RNA 随机引物 PCR 方法检测乳中乳球菌混合培养物转录谱。热处理广泛地用于降低乳的污染，而在贮藏过程中 CO_2 能防止细菌生长并维持乳的品质。 干酪加工过程中或者加工前的这些处理，能够改变发酵剂的代谢活动。 基因表达的变化可以通过不同的显示方法来评价，因此可以通过转录谱的变化来评价不同因素对细菌代谢活动的影响。 本研究旨在建立荧光 RNA 随机引物 PCR (RAP-PCR) 方法来评价乳 CO_2 酸化、皱胃酶浓度、盐浓度对发酵剂基因表达的影响。 与参照条件相比，热处理、伴随着不同中和处理过程的 CO_2 的酸化均使发酵剂基因转录水平受到了影响。 因此 CO_2 酸化后，简单酸中和不能充分地恢复基本的转录水平。 在 Cheddar 干酪制作过程中的标准条件的基础上，随着 NaCl 浓度的增加，或者皱胃酶活性的降低，发酵剂的基因转录出现了重要的改变。 皱胃酶活性的增加导致了发酵剂 RNA 谱发生小的变化。 荧光 RAP-PCR 为更好地理解干酪制作过程中混合发酵剂的基因表达谱的变化，提供了好的方法。

***Lactococcus* / 发酵剂 / 乳制品 / CO_2 / RAP-PCR / 转录谱**

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Résumé – Analyse du profil transcriptomique d'une culture mixte de lactocoques dans le lait par RAP-PCR fluorescente. Pour réduire la contamination microbienne du lait, un traitement thermique est généralement appliqué et une dissolution de CO_2 peut être utilisée pour ralentir la prolifération des bactéries et augmenter la durée de conservation du lait. Ces traitements appliqués avant ou pendant la fabrication fromagère peuvent modifier l'activité métabolique du ferment. Les méthodes d'affichage différentiel révèlent les changements dans l'expression des gènes, donc les effets sur l'activité métabolique peuvent être estimés par la variation des profils de transcription. Cette étude avait pour but de développer la méthode RAP-PCR fluorescente pour évaluer l'influence de trois facteurs sur l'expression des gènes du ferment : l'acidification du lait par le CO_2, la concentration en présure et la concentration en sel. La comparaison des profils de transcription avec un ferment cultivé en conditions de référence a montré que l'expression des gènes était influencée par un traitement thermique excessif et par une acidification par ajout de CO_2 suivie d'une étape de neutralisation. Ainsi, une simple neutralisation de l'acide présent après l'acidification d'un lait par l'ajout de CO_2 n'était pas suffisante pour rétablir le profil de transcription identique au profil de référence. Par comparaison aux conditions de références rencontrées lors de la fabrication d'un fromage de type Cheddar, le profil de transcription du ferment apparaissait principalement influencé par une forte concentration de NaCl et une faible activité de la présure. Une forte activité de la présure n'avait qu'un faible effet sur le profil des ARN du ferment. La RAP-PCR fluorescente est une technique prometteuse pour obtenir une meilleure compréhension de la transcription globale des gènes de cultures mixtes durant une fermentation fromagère.

***Lactococcus* / ferment / produit laitier / dioxyde de carbone / RAP-PCR / transcriptome**

1. INTRODUCTION

During Cheddar cheese making, starter cultures produce enzymes responsible for acidification, proteolysis and metabolite production. These enzymes will influence the organoleptic characteristics of the fermented product. Numerous parameters determine the stability and success of fermentations by affecting the metabolic activity of the microorganisms, including, for example, milk contaminants such as antibiotics and bacteriophages. Thermal or CO_2 treatment of milk, minerals and rennet could affect milk components and thus modify bacterial metabolic activity resulting in a potential fermentation deviation leading to variations in cheese quality.

Monitoring the metabolic activity of strains and starters is most commonly done by pH measurement and lactic acid determination during fermentation. Individual biochemical tests do not describe the entire enzymatic activity of starters, where from 1000 to 2800 different proteins can be synthesized during fermentation [13]. Thus, variation in pH kinetics does not reveal deviations in other enzymatic activities that could affect cheese quality, which is usually determined by sensory evaluation and analytical tests after the costly ripening process. Each enzyme results from mRNA translation, so metabolic activity should be correlated with gene expression by the culture. Our hypothesis is that milk treatments which alter starter activity during fermentation could be detected by comparison of RNA profiles. Microarrays [26], which analyze whole transcriptomes, can be applied when the genome is known. However, if the genome has not been sequenced, the techniques of differential display [30, 31] can be applied to evaluate modifications in transcriptomes according to various conditions either in eucaryotes [10, 29] or in procaryotes [32]. The two molecular techniques of cDNA amplified fragment length polymorphism (cDNA-AFLP) [1] and RNA arbitrarily primed-polymerase chain reaction (RAP-PCR) [32] are the most frequently used for evaluating modifications in transcriptomes [15]. However, cDNA-AFLP requires restriction sites so

Table I. pH of thermal treated milk during fermentation using defined mixed starter culture at the T1 and T2 time points of the modified Pearce test.

Abbreviation	Milk types and thermal treatment	pH ± SD*	
		T1	T2
SMPA10	Skim milk powder autoclaved 10 min at 120 °C	5.31 ± 0.07	4.81 ± 0.08
SMPA15	Skim milk powder autoclaved 15 min at 120 °C	5.08 ± 0.05**	4.78 ± 0.04
SMPP	Skim milk powder pasteurized 30 min at 65 °C	5.16 ± 0.07	4.74 ± 0.07
UHT	Commercial milk treated 2 s at 140 °C	5.46 ± 0.16	4.73 ± 0.06
MF	Commercial microfiltered milk	5.40 ± 0.11	4.70 ± 0.09

* SD is standard deviation.
** $P < 0.05$ within each time point.

transcripts without correct sites will not be revealed at all, while RAP-PCR uses acrylamide electrophoresis that lacks the resolution necessary to efficiently separate amplimers [2]. Fluorescent RAP-PCR or FRAP-PCR [4] uses fluorescent primers and an automated capillary sequencer instead of acrylamide electrophoresis. The differentially expressed amplimers are not identified directly [11, 27], but they can be separated without ambiguity and increase the throughput of differential display analysis [8], while allowing statistical comparison of profiles. The aim of this study was to investigate the effect of CO_2 acidification of milk, NaCl and rennet on global starter activity by comparing transcriptome profiles obtained by FRAP-PCR. Reference conditions for standardizing the comparison of transcriptome profiles were determined by studying the influence of milk preparation (whole milk versus skim milk powder, SMP) and thermal treatment (UHT, microfiltration, pasteurization and autoclaving).

2. MATERIALS AND METHODS

2.1. Strains and culture conditions

The defined mixed starter culture consisted of three *Lactococcus lactis* subsp. *cremoris* strains in equal proportions: LL074, LL225 and LL390 (DSM Food Specialities, Inc., NJ, USA). Two successive cultures inoculated at 1% were incubated at 22 °C in UHT milk, the first for 18 h and the second for 16 h. The final culture was inoculated at 3% in each type of milk to be analyzed (Tab. I) at the T0 time point of the Pearce test (Supplementary Material, Fig. S1, available at www.dairy-journal.org). The incubation parameters of the Pearce test simulate the characteristic temperature and time profile of Cheddar cheese making [19]. Fermentations were done in triplicate and pH was recorded. When required, $CaCl_2$ at the final concentration of 0.2 $g{\cdot}L^{-1}$ and rennet were added at T0. Rennet (Chymax, Fromagex, Rimouski, QC, Canada) was applied at low, standard or high concentrations: 0.04, 0.08 or 0.12 $g{\cdot}L^{-1}$, respectively. NaCl was added at the T4 time point at one of the three concentrations: 15 $g{\cdot}L^{-1}$ (low), 22.5 $g{\cdot}L^{-1}$ (standard) or 25 $g{\cdot}L^{-1}$ (high). For each concentration of rennet tested, only the standard NaCl concentration was used and for each NaCl concentration tested, the standard rennet concentration was applied.

2.2. Preparation of acidified milk and neutralization

Skim milk was commercially pasteurized milk with 0.1% MF (milk fat). Raw whole milk was obtained from Agropur (Natrel division, QC, Canada) prior to homogenization and then pasteurized at 65 °C for

Table II. Summary of milk acidification and neutralization steps and pH of acidified and neutralized pasteurized whole and skim milk at the T4 time point during the Pearce test.

Abbreviation	Milk type	Acidification (pH 6.2)	Neutralization (pH 6.7)	pH ± SD* at T4 time point
WR	Whole milk reference	None	None	4.77 ± 0.06
WCN	Whole	CO_2	NaOH	5.23 ± 0.28**
WCC	Whole	CO_2	Na_2CO_3	6.21 ± 0.34**
WCA	Whole	CO_2	Stirring at 4 °C	4.79 ± 0.04
WHN	Whole	HCl	NaOH	4.71 ± 0.10
SR	Skim milk reference	None	None	5.35 ± 0.34
SC	Skim	CO_2	None	5.39 ± 0.38
SCA	Skim	CO_2	Stirring at 4 °C	5.16 ± 0.40

* SD is standard deviation.
** $P < 0.05$ (comparing pH values of five pasteurized whole milk samples only).

30 min. The same day, separate pasteurized milk aliquots of 100 mL were mixed with filtered CO_2 gas (pores: 0.2 µmol·L^{-1}) until pH 6.2. This acidification led to a CO_2 concentration of around 30.0–38.6 mmol·L^{-1} [12, 14]. As a comparative control, milk acidification was also performed with 5 mol·L^{-1} HCl until the pH attained 6.2. As a pH of 6.7 is necessary in order to ensure high-quality cheese, acidified milk must be neutralized before cheese making. So in this study, acidified milk was neutralized in three ways until pH returned to the reference value of 6.7, by adding 5 mol·L^{-1} of NaOH or Na_2CO_3 powder (0.8 ± 0.2 g for 100 mL of milk) or by degassing with agitation at 4 °C (Tab. II). After acidification and neutralization, each milk treatment was then inoculated at 3% with defined mixed starter at the T0 time point of the Pearce test.

2.3. Cell harvesting

Seven-milliliter samples withdrawn at either T1 and T2, or T4 or T5 of the Pearce test were mixed in equal ratio with RNAProtect® (Qiagen, Mississauga, ON, Canada). Centrifugation at 4500× *g* (no holding time) was first applied to separate the protein debris (casein micelles), while the liquid containing the bacteria was transferred to a new tube for a centrifugation at 16 000× *g* (no holding time). Lipids (from milk fat) were rapidly removed from the tube top with a brush and the liquid phase discarded. The pellet was suspended in 2 mL of RNAProtect® in a 2-mL screw-capped tube and incubated for 5 min at room temperature. After centrifugation at 20 000× *g* (no holding time), the floating material was discarded by inversion, and the pellet was washed in the same tube with 1.5 mL of RNAProtect® followed by centrifugation at 20 000× *g* (no holding time).

2.4. Cell lysis and RNA purification

After evacuating the RNAProtect®, the cell pellet was suspended in 500 µL of lysis buffer (100 g·L^{-1} lysozyme and 10% sucrose, pH 5) and incubated for 5 min at 46 °C. A volume of 1 mL of Trizol® (Invitrogen, Burlington, ON, Canada) at 46 °C, 200 µL of chloroform and 100 µL of β-SDS (10% sodium dodecyl sulfate and 1% β-mercaptoethanol) were added successively and mixed. After incubation for 5 min at 46 °C, the phases were blended by agitation then separated by centrifugation in a precooled centrifuge (20 000× *g* for 5 min at 4 °C).

One milliliter of the aqueous phase was mixed with 500 µL of isopropanol at room temperature and then passaged twice on an RNeasy® column (Qiagen, Mississauga, ON, Canada) by centrifugation at 14 000× *g* for 15 s. The manufacturer's protocol was followed for washing and on-column DNase treatment, except that 33 units of SUPERaseIn™ (Ambion, Applied Biosystems, Foster City, USA) were added. The RNA elution was carried out on ice with 10 µL of RNase-free water. The RNA concentration was quantified at 260 nm with a NanoDrop™ 1000 (Thermo Fisher Scientific, Wilmington, USA) and adjusted to a final concentration of 100 ng·µL^{-1} with RNase-free water (Qiagen, Mississauga, ON, Canada).

2.5. Fluorescent RAP-PCR

Primer design is described in the Supplementary Material, Sections 1 and 2. RNA was used at a concentration of 15 ng·µL^{-1} with 19 units of SUPERase-In and 2.5 µmol·L^{-1} of an equal ratio of primers ST1–ST13 (Supplementary Material, Tab. SI). The mixture was incubated at 65 °C for 5 min then immediately placed on ice for 1 min. The final volume of the retrotranscription reaction was 19 µL and contained: 2 mmol·L^{-1} of total dNTPs, 190 units of SuperScript™ III (Invitrogen, Burlington, ON, Canada), 3.7 µL of 5 X buffer and 5 mmol·L^{-1} of DTT (dithiothreitol). The reverse transcription reactions were incubated at 25 °C for 10 min. The polymerization step was carried out at 45 °C for 2 h, and a final reverse transcriptase inactivation step was applied at 70 °C for 15 min.

A quantity of 20 ng of cDNA was used as template for PCR amplification. For 20 µL of final volume, each reaction mixture contained: 1.67 mmol·L^{-1} of $MgSO_4$, 700 µmol·L^{-1} of total dNTPs, 1.34 units of Hot Start Kod polymerase (EMD Biosciences, Inc., Novagen®, Madison, WI, USA), 2 µmol·L^{-1} of one nonlabeled primer and 2 µmol·L^{-1} of one fluorescent primer. The first step was 95 °C for 10 min for RNA degradation and Hot Start polymerase activation. The second step was a low stringency annealing at 35 °C for 40 min, enabling saturation of all the potential hybridizing sites [30]. The third step was a final elongation of the second strand cDNA at 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; and 72 °C, 60 s. The PCR amplification was mixed with 500 µL of TE (10 mmol·L^{-1} Tris and 1 mmol·L^{-1} EDTA, pH 8) and loaded on a Microcon® YM100 (Millipore, Billerica, MA, USA). The amplicons were washed four times with successive passages of 500 µL of TE on the same column. Elution was carried out with 50 µL of TE, and PCR products were quantified by spectrophotometry at 260 nm.

2.6. Separation by polyacrylamide gel electrophoresis

PCR amplifications (3 µg) were separated on 6% polyacrylamide gels (acrylamide:bisacrylamide with a 29:1 ratio). Electrophoresis was run at 100 V for 24 h at 4 °C. Ethidium bromide was used for staining DNA, which was visualized by UV transillumination.

2.7. Amplicon separation by capillary electrophoresis

A quantity of 75 ng of purified amplicons was mixed with 10 µL of formamide and 0.3 µL of MapMarker® 1000 (Bio-Ventures, Murfreesboro, TN, USA). The mixture was heated at 99 °C for 5 min then cooled on ice for 1 min and injected (injection voltage: 1 kV, injection duration: 30 s) in the ABI Prism® 3100 Genetic Analyzer (Applied Biosystems, Foster City, USA).

Separation was performed using capillaries filled with POP6 polymer under these parameters: run voltage: 15 kV, total

data points for each run: 24 178. The electropherograms were acquired with the GeneMapper® version 3.7 software (Applied Biosystems) under these advanced microsatellite parameters: no smoothing; baseline = 51; peak threshold (blue) = 1; minimum peak half width = 0; polynomial degree search = 5; and window size = 15. To standardize MapMarker® sizing, flag quality was set at low-quality range from 0 to 1E − 6 then pass-range from 1E − 5 to 1. Panel manager marker sizing and microsatellite analysis was applied from 50 to 1500 bases. An AFLP analysis was performed and for each replicate, the size (in bases) and the height (in fluorescent units) of peaks were exported to Excel.

2.8. Statistical analysis

To prevent statistical problems resulting from peak absence of some very small peaks (no height reported), the minimum height of the peaks detected in the run (usually from 1 to 10 under a 6000 scale graduation) was substituted for each of the peaks not detected with GeneMapper (around 1% of the peaks were not detected by the software). Height data were transformed by Neperian logarithm to linearize peak distribution then standardized with AMIADA [33] using the sum of all the heights. Standardization was carried out to a null average and a unitary standard deviation, allowing comparisons among replicates. For each sample withdrawn at one time point, the resulting eight electropherograms were concatenated under Excel® (for milk thermal treatments, the electropherograms from each time point were also concatenated).

Hierarchical clustering (HC) and principal component analysis (PCA) were carried out using "R" statistical software. Euclidean distance was used as the metric because it is a direct similarity measure, allowing rapid and intuitive interpretation of information produced (equation (1)). HC used average linkage and was computed with a bootstrap of 10 000 permutations. PCA and statistical significance (using ANOVA and Tukey honest significant differences) were computed with the "R" package. The number of differentially expressed peaks was identified using the SAM algorithm (FDR = 0.4, 10 000 permutations) from the MEV 4 software [24] and divided by the total number of peaks:

$$\text{Similarity}_{A/B} = \frac{1}{1 + \text{Distance}_{A \to B}}$$
$$\text{Distance}_{A \to B} = \sqrt{2(1 - \text{Similarity}_{A/B})}. \quad (1)$$

3. RESULTS AND DISCUSSION

The mixed starter culture responded to the state of milk nutrient accessibility by adapting their global RNA profiles. The bacterial transcriptome is composed mainly of rRNA and tRNA, while the fraction of mRNA is low (around 5%). Fragments generated by FRAP-PCR can thus be attributed to all RNA transcripts. While many different individual mRNA molecules are represented in the transcriptome (high complexity), the multiple copies of rRNA and tRNA are of similar sequence (low complexity), and thus the same rRNA amplicons will migrate to the same distance. This will reduce its effect on the overall profile composed of 1000 peaks, as long as primers and reaction components are present in sufficient quantity to avoid depletion.

3.1. Effect of milk type and treatment on defined mixed starter transcription profiles by FRAP-PCR

In order to determine the reproducibility of the method and to select reference conditions, different milk types and thermal treatments were first compared. The technique showed high reproducibility, as each peak

of electropherograms was present in triplicate experiments (Supplementary Material, Fig. S2) and replicates were grouped by HC (Supplementary Material, Fig. S3) as well as by PCA (Supplementary Material, Fig. S4). Cluster separation could be attributed to milk type used during the fermentation even if pH could not differentiate most of the milk types (Tab. I). Clusters of electropherograms coming from cultures in microfiltered, pasteurized and UHT milk were located near to one another and separated from the profiles obtained with autoclaved SMP. The first two components of PCA (Supplementary Material, Fig. S3) explain > 50% of the total variance. The first component, explaining nearly 40% of the variance, separated autoclaved from nonautoclaved SMP, whereas the second component divided SMP autoclaved for 10 or 15 min.

Pasteurization and microfiltration are not damaging for milk constituents as there are few physicochemical changes compared to raw milk [20, 25], but milk contains various active RNases [16] and could have microbial contaminants [18]. The RNases could alter starter RNA during extraction and the microbial contaminants could be cocultivated with starter (especially during the two subcultures) and these will give irreproducible RNA profiles. The autoclaving of milk inactivates RNases and kills all microbial contaminants, but the results of this study show that autoclaved SMP induced significant transcriptome changes in the starter compared to microfiltered milk. The autoclaved SMP is not recommended for starter activity study for two reasons: (i) the starter RNA profile of autoclaved SMP was very different from the same SMP treated by pasteurization; (ii) with only 5 min difference between them, the two autoclaving times induced significant starter RNA modifications, indicating a potential deviation of starter activity if the autoclaving of milk is not perfectly time controlled (e.g. cooling time).

Starter fermenting UHT or pasteurized SMP has nearly the same RNA profile as in microfiltered milk, so these two milk types did not notably influence gene transcription. SMP has well-known solubilization problems and so could lead to modification of water activity between different experiments. UHT milk was the most suitable milk for starter gene transcription study, as there was no need of solubilization, no RNA or bacterial contaminants [3], and milk RNases are inactivated by the high temperature reached (around 140 °C) [16]. The UHT treatment did not influence the starter transcriptome as much as autoclaved SMP, so this high temperature treated milk could be used for preparing starter cultures for RNA study of cheese fermentation without important risk of contamination.

3.2. Effect of CO_2 acidification and neutralization on transcription profiles of defined mixed starter cultivated in whole milk

Oxygen displacement by carbon dioxide prevents the growth of aerobic bacteria, but anaerobic bacteria can also be affected by CO_2 [22]. Milk carbon dioxide dissolution leads to a rapid drop of pH caused by carbonic acid formation in milk aqueous phase, but this important acid production is not the only cause of bacterial growth inhibition [9]. Carbon dioxide has important effects on bacterial membrane permeability [7] but more complex effects have been studied, such as interference with bacterial metabolism leading to changes in carbohydrate utilization [17], lowering intracellular pH [7] and varying enzyme activities [6] (such as extracellular lipase [23]). All these combined effects induce a stress that decreases bacterial multiplication and acidification rate.

Samples were taken at the T4 time point, and the pasteurized whole milk cluster was used as control to discover the influence

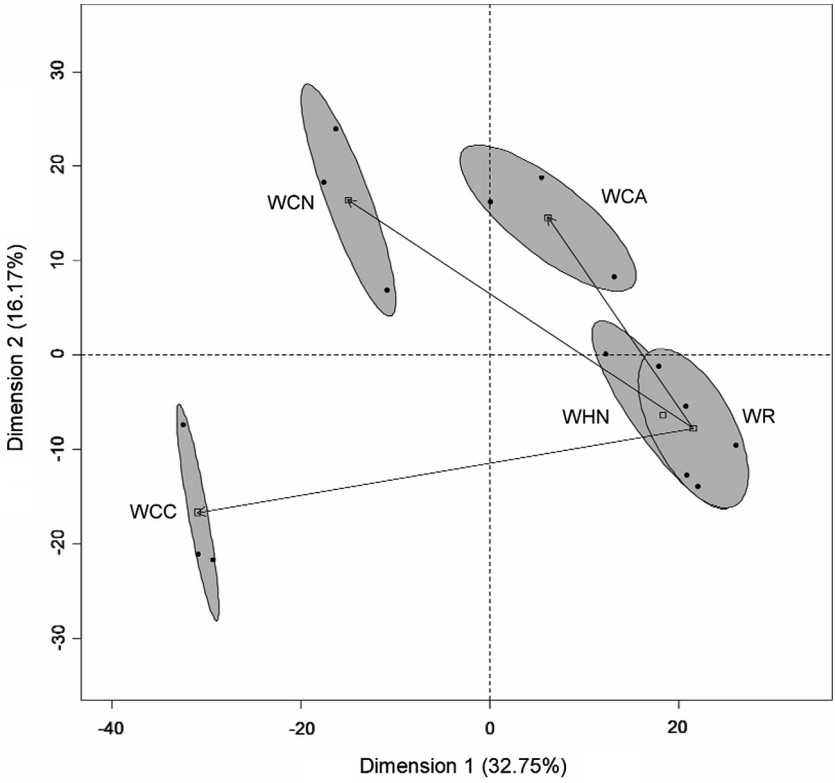

Figure 1. PCA of fluorescent RAP-PCR amplicon profiles obtained from RNA extracted from defined mixed culture fermentation of pasteurized whole milk pretreated with different acidification and neutralization procedures. WR is the pasteurized whole milk reference profile; WCN is whole milk acidified with CO_2 and neutralized with NaOH; WCC is whole milk acidified with CO_2 then neutralized with Na_2CO_3; WCA is whole milk acidified with CO_2 and neutralized by agitation; and WHN is whole milk acidified with HCl and neutralized with NaOH. Gray shading represents the clustering of the three experimental repetitions (●) inside an ellipse which represents a confidence level of 95%. (□) Cluster's barycenter.

of the acidification and neutralization procedures (Tab. II) on RNA profiles. The pH was significantly higher at T4 in CO_2-acidified milk that was neutralized by NaOH or Na_2CO_3 than after the other treatments of pasteurized whole milk. The first two components of PCA totalized around 50% of the variance (Fig. 1). The first component (32.75% of the variance) separates CO_2-acidified milk from pasteurized milk and HCl-acidified milk. The second component explained 16.17% of the variance and could also be associated with CO_2 treatment, as the CO_2 profiles are further from the central axis than those of pasteurized and HCl-acidified milk.

Compared to pasteurized whole milk, the RNA profile of starter fermenting CO_2-acidified whole milk which was neutralized by NaOH or Na_2CO_3 showed important modifications. The RNA profile of starter fermenting HCl-acidified whole milk coupled with NaOH for the neutralization did not show any statistical difference from

whole milk, suggesting that it was not the acidification and NaOH neutralization steps that were causing RNA profile modifications. Therefore, milk acidification by CO_2 could modify RNA profiles of the mixed culture. The type of neutralization step also influenced gene transcription. The cluster of electropherograms from starter cultivated on CO_2-acidified and Na_2CO_3-neutralized whole milk was the farthest removed from the whole milk profile, indicating more extensive modification of the starter RNA profile. Furthermore, this type of neutralization led to important pH buffer action, which could introduce significant delays in acidification needed for cheese manufacturing. Carbon dioxide dissipation by agitation seems a good technique for neutralization to regain similar reference starter activity because this treatment led to only small starter RNA changes.

3.3. Effect of CO_2 acidification and neutralization on transcription profiles of defined mixed starter cultivated in skim milk

Pasteurized skim milk without CO_2 treatment or neutralization was used as the fermentation reference. Two different treatments were applied to pasteurized skim milk: acidification with CO_2 to pH 6.2 without neutralization and acidification with CO_2 to pH 6.2 followed by neutralization by agitation at 4 °C. Experiments using pasteurized whole milk were repeated for a reference profile among the various replicates. The PCA results (Fig. 2) show that FRAP-PCR profiles from mixed cultures in skim milk with different carbonation or agitation treatments were separated from those of pasteurized whole milk along the first component. The PCA shows clustering of all skim milk samples into one group, whereas treated whole milks were well separated from one another. All the FRAP-PCR profiles from skim milk were electrophoresed on a 6% acrylamide gel (Supplementary Material, Fig. S5). For each primer pair, no differences between the band profiles could be detected. Thus, CO_2 acidification experiments using pasteurized skim milk did not induce detectable modifications in electropherograms obtained either by FRAP-PCR or by polyacrylamide gel electrophoresis. Protein and mineral contents are essentially the same between whole milk and skim milk [21], leading to the conclusion that there was no irreversible action of CO_2 on casein or colloidal calcium phosphate that could influence starter gene transcription during fermentation. Therefore, the fat globules could be the origin of the difference between pasteurized whole milk and skim milk in the effect of CO_2 acidification on starter RNA profiles. Carbon dioxide treatment could lead to imprisonment of carbonated molecules in milk fat globules [28], which could be released during cheese manufacture, leading to interactions with starter bacteria even if the *L. lactis* subsp. *cremoris* strains used were resistant to CO_2 influence [5].

3.4. Rennet and NaCl influence on defined mixed starter transcriptome profiles

At the salt concentrations used in this study, the three individual strains have about the same growth (data not shown). Mixed starter fermented with different NaCl and rennet concentrations did not show any influence on the final pH attained in the Pearce test (Tab. III). Differentially expressed peaks (either activated or repressed compared to the standard condition) were identified by SAM analysis (Tab. IV). The high rennet treatment resulted in the same proportion of activated peaks (i.e. peaks of greater height than in the standard condition) as for repressed peaks, while the low rennet condition showed double the number of repressed peaks compared to activated ones (Tab. IV). High NaCl concentration resulted in almost the same number

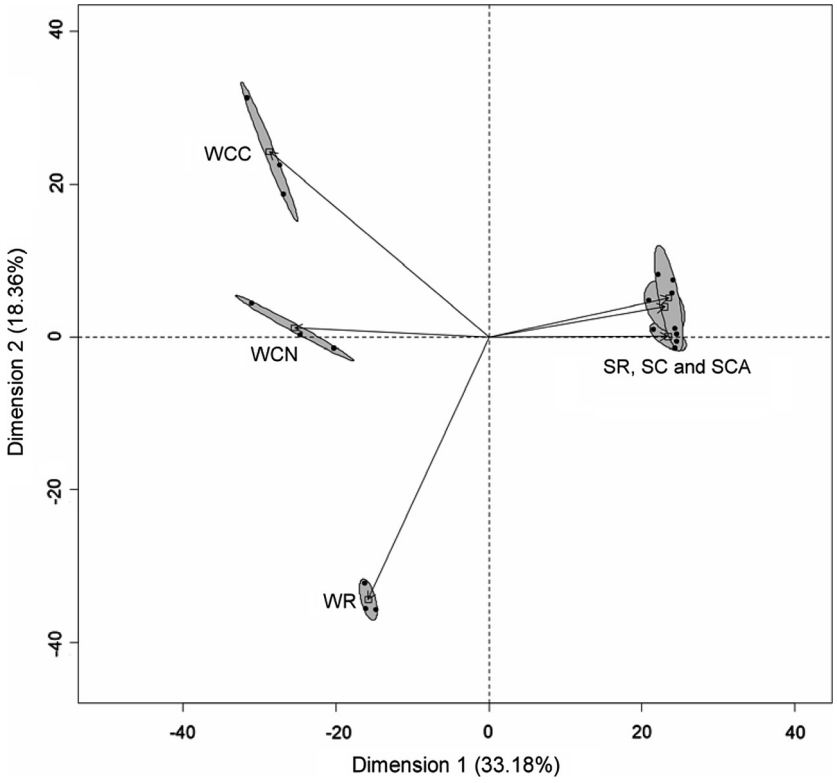

Figure 2. PCA of fluorescent RAP-PCR profiles obtained from RNA extracted from defined mixed culture fermentation of pasteurized whole or skim milk treated by acidification and neutralization. WR is the whole milk reference profile; WCN is whole milk acidified with CO_2 and neutralized with NaOH; WCC is whole milk acidified with CO_2 then neutralized with Na_2CO_3; SR is pasteurized skim milk reference with no treatment; SC is pasteurized skim milk treated with CO_2; and SCA is pasteurized skim milk treated with CO_2 then neutralized by agitation. Gray shading represents the clustering of the three experimental repetitions (●) inside an ellipse which represents a confidence level of 95%. (□) Cluster's barycenter.

Table III. pH of UHT milk at T5 (1 h and 45 min after the T4 time point) of the modified Pearce starter activity test.

Condition	NaCl ($g \cdot L^{-1}$)	Rennet ($g \cdot L^{-1}$)	pH ± SD*
Standard condition	22.5	0.08	5.10 ± 0.06
High rennet	22.5	0.12	5.14 ± 0.05
Low rennet	22.5	0.04	5.00 ± 0.06
High NaCl	25	0.08	5.11 ± 0.05
Low NaCl	15	0.08	5.06 ± 0.06

* SD is standard deviation.

Table IV. Number of peaks differentially expressed for each condition compared to standard conditions identified using the SAM algorithm (FDR = 0.4%, 10 000 permutations) of the MEV 4.0 software. Percentage of the total is reported in parentheses.

Condition	Activated peaks	Repressed peaks
High rennet	22 (2%)	17 (2%)
Low rennet	18 (2%)	50 (4%)
High NaCl	42 (4%)	28 (2%)
Low NaCl	8 (1%)	50 (4%)

of activated peaks as the number of repressed peaks identified when low NaCl concentration was used.

The PCA clusters are separated by the two components simultaneously, indicating that each of the two first eigenvectors was a combination of NaCl and rennet influence (Fig. 3). These eigenvectors were nearly equal in the variance explained (24.14% for the first and 16.25% for the second). The profiles obtained from the RNA extracted from mixed cultures with low rennet concentration were the farthest from the standard cluster near the center and showed the highest positive vector in the first dimension. Comparison with the reference cluster shows that both the salt and rennet vectors (high and low) are nearly opposite in direction, and are in opposing quadrants, indicating opposing correlation of the variance caused by condition-specific peaks. The cluster corresponding to the standard NaCl concentration was equally centered between the two profile clusters representing low and high NaCl concentrations, even if the concentration gradient varied (7.5 $g \cdot L^{-1}$ between low and standard compared to only 2.5 $g \cdot L^{-1}$ between standard and high concentration). This indicates an important influence on the transcriptome caused by NaCl addition and less difference when NaCl decreased. As the vector of the clusters is equally distanced from both principal components, this indicates a combination of the influence of both NaCl and rennet. Rennet concentration thus had an opposite effect from NaCl, as the cluster of profiles from the lowest rennet concentration was at a greater distance from the profile obtained with the standard concentration of rennet. This indicates that gene transcription of the starter was greatly modified by low rennet condition and less by high rennet addition.

4. CONCLUSION

RNA profiles of starter fermenting CO_2-acidified milk were modified by CO_2 molecules presumably dissolved in fat globules and slowly released. Agitation was more effective than NaOH or Na_2CO_3 for neutralization to return the profile to that obtained in whole milk. FRAP-PCR was able to show that starter gene transcription was more influenced by an increase in NaCl compared to a decrease in NaCl. Rennet at a high concentration had only a little influence on gene transcription, but low rennet activity had great influence. This indicates that an increase in NaCl must be compensated by an increase in rennet. For this starter, rennet activity appeared to be optimal at standard concentration.

This is the first report of the application of FRAP-PCR to the study of starter activity in cultures emulating cheese fermentation. This method could be used to determine the level of influence of parameters and help to understand the way changes in Cheddar cheese fermentation procedures could interfere with global gene transcription of the starter. By determining starter gene responses during Cheddar cheese manufacturing, optimal

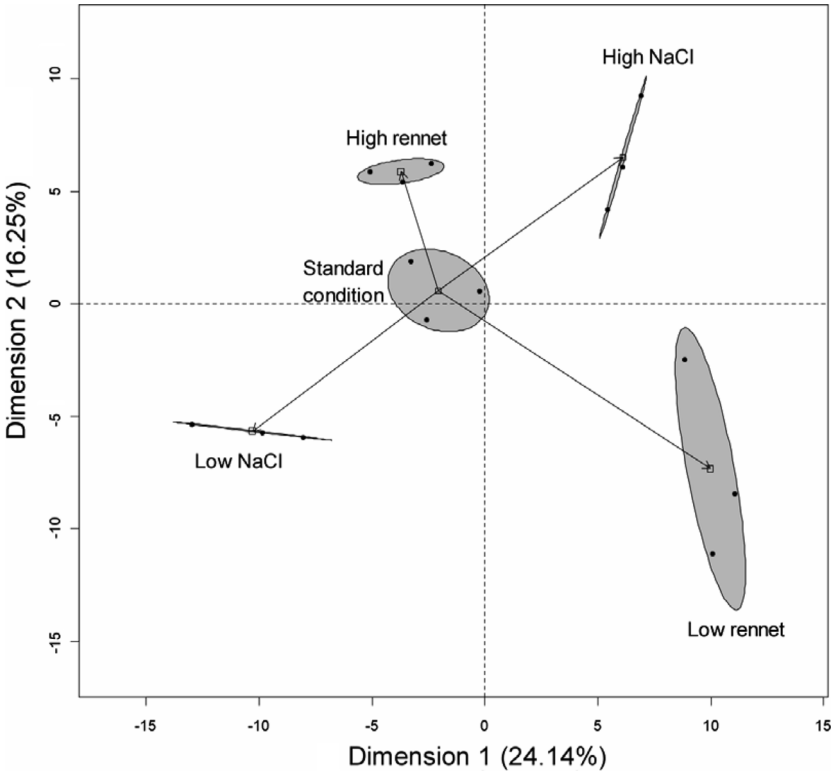

Figure 3. PCA of fluorescent RAP-PCR profiles obtained from RNA extracted from fermentation with low, standard and high concentrations of rennet and NaCl (as listed in Sect. 2). Gray shading represents the clustering of the three experimental repetitions (●) inside an ellipse which represents a confidence level of 95%. (□) Cluster's barycenter.

cheese fermentation could be predicted by comparison with a reference such as a cheese with an excellent grade. Another potential of FRAP-PCR could be the detection of differentially expressed peaks due to starter expressing phage RNA, thus revealing potential fermentation failure due to phage. This technique could also be used for studying bacterial associations in order to further our understanding of microbial interactions in food matrices.

Acknowledgments: The authors gratefully acknowledge the financial support provided by the Fonds Québécois de recherche sur la nature et les technologies (FQRNT), Novalait, Inc., the Ministère de l'Agriculture, des Pêcheries et de l'Alimentation du Québec (MAPAQ) as well as Agriculture and Agri-Food Canada.

REFERENCES

[1] Bachem C.W., van der Hoeven R.S., de Bruijn S.M., Vreugdenhil D., Zabeau M., Visser R.G., Visualization of differential gene expression using a novel method of RNA fingerprinting based on AFLP: analysis of gene expression during potato tuber development, Plant J. 9 (1996) 745–753.

[2] Bauer D., Muller H., Reich J., Riedel H., Ahrenkiel V., Warthoe P., Strauss M., Identification of differentially expressed

mRNA species by an improved display technique (DDRT-PCR), Nucleic Acids Res. 21 (1993) 4272–4280.

[3] Birlouez-Aragon I., Sabat P., Gouti N., A new method for discriminating milk heat treatment, Int. Dairy J. 12 (2002) 52–57.

[4] Crump D., Chiu S., Trudeau V.L., Kennedy S.W., Fluorescent RNA arbitrarily primed polymerase chain reaction. A new differential display approach to detect contaminant-induced alterations of gene expression in wildlife species, in: Cristofre Martin C. (Ed.), Methods in Molecular Biology: Environmental Genomics, Vol. 410, Humana Press Inc., Totowa, USA, 2008, pp. 15–27.

[5] Enfors S.O., Molin G., Effect of high concentrations of carbon dioxide on growth rate of *Pseudomonas fragi*, *Bacillus cereus* and *Streptococcus cremoris*, J. Appl. Bacteriol. 48 (1980) 409–416.

[6] Gill C.O., Tan K.H., Effect of carbon dioxide on growth of *Pseudomonas fluorescens*, Appl. Environ. Microbiol. 38 (1979) 237–240.

[7] Hon S.I., Pyun Y.R., Membrane damage and enzyme inactivation of *Lactobacillus plantarum* by high pressure CO_2 treatment, Int. J. Food Microbiol. 63 (2001) 19–28.

[8] Jones S.W., Cai D., Weislow O.S., Esmaeli-Azad B., Generation of multiple mRNA fingerprints using fluorescence-based differential display and an automated DNA sequencer, Biotechniques 22 (1997) 536–543.

[9] King J.S., Mabbitt L.A., Preservation of raw milk by addition of carbon dioxide, J. Dairy Res. 49 (1982) 439–447.

[10] Liang P., Pardee A.B., Differential display of eukaryotic messenger RNA by means of the polymerase chain reaction, Science 257 (1992) 967–971.

[11] Luehrsen K.R., Marr L.L., van der Knaap E., Cumberledge S., Analysis of differential display RT-PCR products using fluorescent primers and GENESCAN software, Biotechniques 22 (1997) 168–174.

[12] Ma Y., Barbano D.M., Effect of temperature of CO_2 injection on the pH and freezing point of milks and creams, J. Dairy Sci. 86 (2003) 1578–1589.

[13] Makarova K., Slesarev A., Wolf Y., Sorokin A., Mirkin B., Koonin E., Pavlov A., Pavlova N., Karamychev V., Polouchine N., Shakhova V., Grigoriev I., Lou Y., Rohksar D., Lucas S., Huang K., Goodstein D.M., Hawkins T., Plengvidhya V., Welker D., Hughes J., Goh Y., Benson A., Baldwin K., Lee J.H., Diaz-Muniz I., Dosti B., Smeianov V., Wechter W., Barabote R., Lorca G., Altermann E., Barrangou R., Ganesan B., Xie Y., Rawsthorne H., Tamir D., Parker C., Breidt F., Broadbent J., Hutkins R., O'Sullivan D., Steele J., Unlu G., Saier M., Klaenhammer T., Richardson P., Kozyavkin S., Weimer B., Mills D., Comparative genomics of the lactic acid bacteria, Proc. Natl. Acad. Sci. USA 103 (2006) 15611–15616.

[14] Martin J.D., Werner B.G., Hotchkiss J.H., Effects of carbon dioxide on bacterial growth parameters in milk as measured by conductivity, J. Dairy Sci. 86 (2003) 1932–1940.

[15] Meade J.D., Cho Y.J., Fisher J.S., Walden J.C., Guo Z., Liang P., Automation of fluorescent differential display with digital readout, in: Liang P., Meade J.D., Pardee A.B. (Eds.), Methods in Molecular Biology: Differential Display Methods and Protocols, Vol. 317, Humana Press Inc., Totowa, USA, 2006, pp. 23–57.

[16] Meyer D.H., Kunin A.S., Maddalena J., Meyer W.L., Ribonuclease activity and isoenzymes in raw and processed cows' milk and infant formulas, J. Dairy Res. 70 (1987) 1797–1803.

[17] Molin G., Effect of carbon dioxide on growth of *Pseudomonas putida* ATCC 11172 on asparagine, citrate, glucose, and lactate in batch and continuous culture, Can. J. Microbiol. 31 (1985) 763–766.

[18] Nielsen E.W., Principles of cheese production, in: Hui Y., Meunier-Goddik L., Hansen A., Josephsen J., Nip W.-K., Stanfield P., Toldrá F. (Eds.), Handbook of Food and Beverage Fermentation Technology, Marcel Dekker, New York, USA, 2004, pp. 219–239.

[19] Pearce L.E., Activity tests for cheese starter cultures, New Zealand, J. Dairy Technol. 4 (1969) 246–247.

[20] Pedersen P.J., Microfiltration for the reduction of bacteria in milk and brine, new applications of membrane processes, Int. Dairy Fed. Special Issue No. 9201 (1992) 33–50.

[21] Posati L.P., Orr M.L., Composition of foods: dairy and egg products; raw, processed, prepared, Agriculture Handbook No. 8-1, Agricultural Research Service, USDA, Washington, USA, 1976.

[22] Roberts R.F., Torrey G.S., Inhibition of psychrotrophic bacterial growth in refrigerated milk by addition of carbon dioxide, J. Dairy Sci. 71 (1988) 52–60.

[23] Rowe M.T., Effect of carbon dioxide on growth and extracellular enzyme production by *Pseudomonas fluorescens* B52, Int. J. Food Microbiol. 6 (1988) 51–56.

[24] Saeed A.I., Sharov V., White J., Li J., Liang W., Bhagabati N., Braisted J., Klapa M., Currier T., Thiagarajan M., Sturn A., Snuffin M., Rezantsev A., Popov D., Ryltsov A., Kostukovich E., Borisovsky I., Liu Z., Vinsavich A., Trush V., Quackenbush J., TM4: a free open-source system for microarray data management and analysis, Biotechniques 34 (2003) 374–378.

[25] Scaloni A., Perillo V., Franco P., Fedele E., Froio R., Ferrara L., Bergamo P., Characterization of heat-induced lactosylation products in caseins by immunoenzymatic and mass spectrometric methodologies, Biochim. Biophys. Acta 1598 (2002) 30–39.

[26] Schena M., Shalon D., Davis R.W., Brown P.O., Quantitative monitoring of gene expression patterns with a complementary DNA microarray, Science 270 (1995) 467–470.

[27] Sturtevant J., Applications of differential-display reverse transcription-PCR to molecular pathogenesis and medical mycology, Clin. Microbiol. Rev. 13 (2000) 408–427.

[28] Walstra P., Wouters J.T.M., Geurts T.J., Dairy Science and Technology, CRC Press, Boca Raton, USA, 2006.

[29] Welsh J., Chada K., Dalal S.S., Cheng R., Ralph D., McClelland M., Arbitrarily primed PCR fingerprinting of RNA, Nucleic Acids Res. 20 (1992) 4965–4970.

[30] Welsh J., McClelland M., Fingerprinting genomes using PCR with arbitrary primers, Nucleic Acids Res. 18 (1990) 7213–7218.

[31] Williams J.G., Kubelik A.R., Livak K.J., Rafalski J.A., Tingey S.V., DNA polymorphisms amplified by arbitrary primers are useful as genetic markers, Nucleic Acids Res. 18 (1990) 6531–6535.

[32] Wong K.K., McClelland M., Stress-inducible gene of *Salmonella typhimurium* identified by arbitrarily primed PCR of RNA, Proc. Natl. Acad. Sci. USA 91 (1994) 639–643.

[33] Xia X., Xie Z., AMADA: analysis of microarray data, Bioinformatics 17 (2001) 569–570.