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Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria metabolic activity until the end of the ripening of Emmental cheese

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ABSTRACT

For Emmental manufacture two kinds of adjunct culture are added: (i) thermophilic lactic acid bacteria (starters) such as *Lactobacillus helveticus* (LH), and *Streptococcus thermophilus* (ST) growing the first day of the manufacture and (ii) ripening culture. ST and LH have a key role in curd acidification and proteolysis at the beginning of the manufacture but are considered to be lyzed for a great part of them at the ripening step. The aim of this work was to assess the metabolic activity of these bacteria throughout manufacture and ripening. During Emmental cheesemaking, LH and ST were subjected to i) population quantification by numerations and by quantitative PCR (qPCR) ii) reverse transcription (RT) Temporal Temperature Gel Electrophoresis (TTGE) iii) transcript quantification by RT-qPCR targeting 16S rRNA, *tuf* and *groL* mRNAs to evaluate bacterial metabolic activity. During ripening, ST and LH numerations showed a 2.5 $\log_{10}$ loss of culturability whereas qPCR on pelleted cells revealed only one $\log_{10}$ of decrease for both of these species. 10^9 ST and 10^8 LH cells/g of cheese still remained. They contained a stable number of 16S transcript and at least 10^6 copies of mRNAs per 10^9 cells until the end of ripening. These results prove the unexpected persistency of thermophilic lactic acid bacteria starters (ST and LH) metabolic activity until the end of ripening and open new perspectives in term of their involvement in the quality of cheeses during ripening.

1. Introduction

Cheese manufacture is largely based on lactic acid bacteria fermentation. They can be added deliberately as starter cultures or are adventitious microflora selected during the fermentation process. Population dynamics and activity associated with biochemical processes are of crucial importance for the development of the unique flavour and texture characteristic of a given cheese type (Thierry et al., 1998; Gagnaire et al., 2004; Fröhlich-Wyder and Bachmann, 2004).

Present knowledge of microbial diversity and dynamics in cheese is mainly based on culture-dependent methods, involving traditional numeration followed by identification of dominant microorganisms using phenotypic and molecular methods (Juste et al., 2008). Using these methods, it has been shown in Swiss-type cheese that starter bacteria decrease in culturability over the ripening period in most cheeses. Lactic acid bacteria thermophilic starters are mainly involved in (i) acidification of curd *via* the fermentation of lactic acid into lactate and (ii) proteolysis essential for the development of the stretching, melting and aromatic properties (Gagnaire et al., 1998; Lortal et al., 1997; Valence et al., 1998). In Emmental cheese, they grow during the pressing step within the first day of manufacture. The counts of *Streptococcus thermophilus* and *Lactobacillus helveticus*, two thermophilic lactic acid bacteria used as starter in Emmental cheese manufacture decrease by 2–7 $\log_{10}$ cfu per gram of cheese over the two months ripening period. This decrease is generally associated with cell lysis releasing intracellular peptidases involved in cheese proteolysis during the ripening (Deutsch et al., 2002; Thierry et al., 1998; Valence et al., 1998). In long ripened Italian cheeses, such as Parmigiano Reggiano, lactic acid bacteria contribute to flavour development during ripening by converting milk proteins into

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peptides and free amino acids successively catabolized into aroma compounds (Lindner et al., 2008).

Molecular methods have enabled to precise importance of each thermophilic bacteria species throughout ripening of cheese. A combination of enumeration step with repetitive sequence PCR applied to different italian commercial ripened cheeses has permit to prove the persistency of thermophilic bacteria species such as *L. helveticus* and *Lactobacillus delbrueckii* (Jensen et al., 2009). Using PCR fingerprinting, applied to Grana cheese, prevalence of *L. delbrueckii* subsp. *lactis* was observed after two months of ripening (Zago et al., 2007). To our knowledge metabollic activity of thermophilic lactic acid bacteria during ripening has never been investigated at species level. In Emmental cheese, the use of RT PCR-TTGE throughout manufacture revealed, for the first time, the persistence of 16S RNA transcript of *S. thermophilus* and *L. helveticus* two months after the decrease of culturability evaluated by plate counting (Parayre et al., 2007). In the same way, in Castelmagno cheese (Dolci et al., 2010), whereas *L. helveticus* was never isolated on selective media, DGGE experiments carried on RNA matrix extracted from ripened cheese revealed that this species is still active. These results suggest that 16S RNA transcripts is either too much stable to provide a dynamic image of the bacterial communities, or is *de novo* synthesized by a sub-population which remains active throughout the ripening.

Different approaches can be used to evaluate bacteria viability status. The measurements of membrane potential, permeability of membrane to dye, and flow cytometry enable the assessment of the cell integrity. The detection of respiration and the measurement of enzymatic activities can also be performed. To our knowledge, these approaches cannot be used to evaluate the activity or the integrity of a given species when it is mixed in a complex ecosystem and in a matrix such as cheese. The presence or the absence of nucleic acids in the cell (PCR, hybridization, staining) can be tested with a bias due to DNA and stable RNA persistence. Dynamic protein synthesis during ripening can be evaluated by pulse chase radioactivity followed by 2D gel electrophoresis. However, all these methods are impossible to implement in a cheese where bacteria are embedded and not accessible to isotope.

RT-qPCR has been first proven to be very useful to evaluate metabolic activity of food pathogenic bacteria. It was successfully applied for example to *Staphylococcus aureus* in meat to evaluate the ability of the bacteria to produce enterotoxin in food (Alarcon et al., 2006) and to *Listeria monocytogenes* in cheese (Rantsiou et al., 2008). Moreover, RT-qPCR revealed the metabolic activity and pathogenicity of *Vibrio cholerae* in contaminated water while this population was unculturable on conventional media (Gonzalez-Escalona et al., 2006). Recently, RT-qPCR was successfully applied to ripened Emmental cheese and confirms the metabolic activity of *Lactobacillus paracasei* and *Propionibacterium freudenreichii*, two ripening species which show a stable culturable population until the end of the ripening (Falentin et al., 2010).

The aim of the present study was to quantify the populations and investigate the metabolic activity of two thermophilic lactic acid bacteria (starters), *S. thermophilus* and *L. helveticus* which both exhibit marked loss of culturability during the ripening of Emmental cheese. In this aim, culture-dependent and culture-independent methods have been used in parallel. Culture-independent methods included PCR-TTGE, RT-PCR-TTGE, qPCR, and RT-qPCR, targeting 16S gene and also more labile gene transcripts (*tuf* and *groL* genes). Results give a new image of starter bacteria dynamics in Emmental cheese and open perspectives in terms of active involvement of thermophilic lactic acid bacteria not only during acidification but also during the ripening as active cells.

2. Material and methods

2.1. Bacterial strains and culture conditions

S. thermophilus ITG ST88 and *L. helveticus* ITG LH56 were used as lactic starters. *P. freudenreichii* ITG P14 and *L. paracasei* ITG LC225 were used as ripening cultures. All cultures used were from Actilait's collection of Emmental cheese bacteria and provided by Laboratoires Standa, Caen, France.

L. helveticus cultures were grown in MRS broth (Becton Dickinson, France) at 43 °C for 2 days without shaking and *S. thermophilus* in M17 broth added with lactose (5 g/L) at 43 °C for 1 day without shaking for DNA extractions according to Deutsch et al. (2002).

2.2. Cheese manufacture

Small scale (1/100) experimental Emmental cheeses were manufactured in triplicate from the same batch of thermized and microfiltered milk according to a standardized cheese-making process previously described (Richoux and Kerjean, 1995; Thierry et al., 2006). The starters were composed of *S. thermophilus* ITG ST88, inoculated at 10^6 colony-forming units (CFU)/g of milk, *L. helveticus* ITG LH56, inoculated at 10^5 CFU/g of milk, *P. freudenreichii* ITG P14, inoculated at 10^5 CFU/g of milk. In addition, *L. paracasei* ITG LC225 was inoculated at 10 CFU/g of milk, a population similar to that of non-starter lactic acid bacteria naturally contaminating cheese milk. Bulk starters were used for *S. thermophilus* (grown on Marstar 412A medium, Danisco, Dangé Saint Romain, France) and *L. helveticus* (grown on Phagex Lb, Laboratoires Standa) whereas *P. freudenreichii* and *L. paracasei* were added to cheese milk as lyophilized cultures to mimic the industrial process. The cheeses were manufactured with 10 L of milk in 10 L cylindrical jacked stainless steel vats (Chalon-Mégard, Cluses, France). After 60 min of incubation at 32 °C, calf rennet was added. When the proper firmness was achieved (measured with the *Gelograph* sensor, Gel Instrument, Thawil, Switzerland), the gel was cut with wires into curd grains of 4 mm mean diameter. After 15 min stirring, the temperature of the whey-curd mixture was raised to at 53.5 °C for 33 min and maintained for 45 min. During this period, stiring continued. The curd was then moulded in 12 cm diameter moulds.

The pressing step (6 h) and the acidification step (14 h) were conducted in thermostated ovens at 48 °C and 36 °C, respectively, in order to mimic the cooling rate of the centre of 80 kg full-size Emmental wheels. Cheeses were then cooled and salted for 5 h in a NaCl-saturated brine at 7 °C, wrapped under vacuum in BK1L-ripening bags (Cryovac, Epernon, France) and ripened at 12 °C for 21 days and then at 24 °C for 28 days. Cheeses were sampled at four manufacture stages and seven ripening stages, as detailed in Table 1.

2.3. Cheese compositional analysis

Samples of ripened cheeses were analysed for moisture, protein, fat, pH and NaCl by classical methods as previously described (Richoux and Kerjean, 1995).

For lactose and D-galactose quantifications, 10 g of cheese were homogenised in 90 ml water in a warring blender, 1 min at low speed followed by 1 min at high speed (Waring Laboratory & Science). After a 10 min centrifugation at 8000 g at 4 °C, supernatant was diluted in water at 1: 10 for D-galactose and D-lactose quantification, except for the milk sample diluted at 1:100 for lactose quantification. Lactose and galactose concentrations were evaluated at each step of cheese manufacture by the UV-method kit