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Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models

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ABSTRACT

The presence of *Lactococcus lactis*, commonly employed as starter culture, was, recently, highlighted and investigated during late cheese ripening. Thus, the main goal of the present study was to assess the persistence and viability of this microorganism throughout manufacturing and ripening of model cheeses. Eight commercial starters, constituted of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*, were inoculated in pasteurized milk in order to manufacture miniature cheeses, ripened for six months. Samples were analysed at different steps (milk after inoculum, curd after cutting, curd after pressing and draining, cheese immediately after salting and cheese at 7, 15, 30, 60, 90, 120, 150 and 180 days of ripening) and submitted to both culture-dependent (traditional plating on M17) and -independent analysis (reverse transcription-quantitative PCR).

On the basis of direct RNA analysis, *L. lactis* populations were detected in all miniature cheeses up to the sixth month of ripening, confirming the presence of viable cells during the whole ripening process, including late stages. Noteworthy, *L. lactis* was detected by RT-qPCR in cheese samples also when traditional plating failed to indicate its presence. This discrepancy could be explain with the fact that lactococci, during ripening process, enter in a stressed physiological state (viable not culturable, VNC), which might cause their inability to grow on synthetic medium despite their viability in cheese matrix. Preliminary results obtained by “resuscitation” assays corroborated this hypothesis and 2.5% glucose enrichment was effective to recover *L. lactis* cells in VNC state.

The capability of *L. lactis* to persist in late ripening, and the presence of VNC cells which are known to shift their catabolism to peptides and amino acids consumption, suggests a possible technological role of this microorganism in cheese ripening with a possible impact on flavour formation.

KEYWORDS

Lactococcus lactis; model cheese; RT-qPCR; viability; ripening; VNC.

INTRODUCTION

Lactococcus lactis have a long history of use in milk fermentation, from small-scale traditional operations to well-controlled industrial applications (Cretenet et al., 2011). The growth of this

microorganism in milk is associated with the rapid production of lactic acid, which contributes to curd formation, prevents the growth of pathogenic and spoilage bacteria and creates optimal biochemical conditions for ripening (Fernández et al., 2011). Carefully selected strains of *L. lactis* are main components of starter cultures for dairy fermentations (Parente and Cogan, 2004) and, thus, it is clear the interest of many authors in the investigating of functional and metabolic gene expression of this microorganism (Cretenet et al., 2011; Dressaire et al., 2008; Fernández et al., 2011; García-cayuela et al., 2012; Taïbi et al., 2011). Although the technological role of this species has been always related to the manufacturing phase, lactococci also contribute to the final texture (moisture, softness) and flavour of dairy products, via their proteolytic and amino acid conversion pathways (Smit et al., 2005).

Recent studies, based on culture-independent analysis, have shown the presence of alive *L. lactis* cells in late ripened dairy products (Desfossés-Foucault et al., 2013; Dolci et al., 2014, 2010; Flórez and Mayo, 2006; Masoud et al., 2011; Rantsiou et al., 2008; Ruggirello et al., 2014). On the other hand, traditional studies based on cultivation of lactic acid bacteria (LAB), on selective media, rarely highlighted the presence of this microorganism in late ripening stages (Dolci et al., 2008; Neviani et al., 2009; Randazzo et al., 2010). These mixed evidences support the thesis of some authors about the existence of cells in a viable but not culturable (VNC) state, which is a current complex and controversial topic (Ganesan et al., 2007; Oliver, 2005; Ruggirello et al., 2014; Stuart et al., 1999). Up to now, no direct approaches to detect and resuscitate metabolically active bacteria, which are not able to form colonies on synthetic media, are available to directly prove the existence of this state; moreover, the molecular events associated with the VNC state are not yet well delineated (Weimer, 2011). Furthermore, it has been proposed that metabolic activity, in intact bacterial cells, defines cell viability, but there are still some challenges regarding dead/viable definitions and discussions concerning metabolic activity in dead cells (Achilleos and Berthier, 2013).

In a recent study, our group optimized a reverse transcription-quantitative PCR (RT-qPCR) protocol to selectively detect alive *L. lactis* in complex cheese matrices (Ruggirello et al., 2014). The absence or low abundance of the microorganism on M17 plates, also when it was detected by RT-qPCR, corroborated the thesis that *L. lactis* starter populations could enter in VNC state during ripening.

Some authors found that carbohydrate starvation, in ripened cheeses, is one of the causes, with osmotic and pH stress, for the loss of lactococci ability to grow and divide on synthetic medium (Stuart et al., 1999; Ganesan et al., 2007; Weimer, 2011).

In the present study, we wanted to investigate further the persistence and viability of eight *L. lactis* commercial starters, throughout cheese manufacturing and ripening. To better assess their behaviour, we used a model cheese, precisely, an Italian Tome-like cheese, and monitored, by both culture-dependent and –independent methods, different steps in cheesemaking process and ripening up to six months. The two approaches were compared in order to evaluate the viability and loss of growth ability on synthetic medium of the different starters. Moreover, in order to support the hypothesis of the possible presence of *L. lactis* cells in VNC state, we tried to resuscitate them by supplying different carbon sources.

MATERIALS AND METHODS

Starter cultures and cheesemaking. The commercial starter cultures used in the present study were purchased from a producing Italian company as lyophilized cells of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*. Precisely, starters coded stM, stC, stJ, stB, stK were constituted of *L. lactis* subsp. *lactis*, starters stX, stY of *L. lactis* subsp. *cremoris*, while a mixture of *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* composed starter stW. They were used as inoculum for cheesemaking of miniature cheeses according to the protocol described by Bertolino et al. (2008).

The absence of contaminating species was verified, for each starter, by DNA extraction from 10 g of lyophilized cultures (Cocolin et al., 2004), followed by amplification of the variable V3 region of 16S rRNA gene (Ampe et al., 1999) and DGGE (Denaturing gradient gel electrophoresis) of the amplicons, according to the protocol described by Cocolin et al. (2004). The presence, in DGGE gels, of a single band, for each starter, co-migrating with *L. lactis* control, confirmed the dominance of *L. lactis* and the absence of other microbial contaminants.

For each production, 200 mL of pasteurized milk were transferred to wide-mouth plastic centrifuge bottles, warmed to 37°C and maintained at this temperature in a water bath. Starters were added,

following the producer's instructions, to the milk and rehydrated for 30 min, $CaCl_2$ (132 µL of a 1 mol/L solution) and rennet (43.5 µL) were then added and the milk held for 40-50 min until a firm coagulum was formed. The coagulum in the bottles was cut manually by cutters made of wires stretched 1 cm apart across a frame. The temperature was then increased to 41 °C over 30 min. Curd and whey were then centrifuged at room temperature for 60 min at 1700xg in centrifuge Megafuge 11R (Thermo Scientific Heraeus, Waltham, MA, USA). The whey was drained and the cheese obtained was inverted in the bottle and held in thermostat at 30 °C for 18h. It was then centrifuged, again, at 1700xg for 20 min, with the top surface of the cheese now placed at the bottom (to obtain a smooth surface on both sides). After further whey drainage, the cheese was brine salted (20 % NaCl, 0.05 % $CaCl_2 \cdot 2H_2O$) for 30 min at room temperature. After salting, the cheese was removed from the bottles, wiped with tissue paper, vacuum packed and ripened at 8 °C for 6 months.

Sampling and pH measurements. For each starter, miniature cheeses (23-25 g), in duplicate, were carried out. The samples were collected at the following steps: milk after inoculum of the starter (M), curd after cutting (CuC), curd after draining (CuD), cheese immediately after salting (ChS) and cheese at 7 (Ch7), 15 (Ch15), 30 (Ch30), 60 (Ch60), 90 (Ch90), 120 (Ch120), 150 (Ch150) and 180 (Ch180) days of ripening. They were analysed for the presence and viability of *L. lactis* by both culture-dependent and -independent methods.

The pasteurized milk was checked, before starter inoculum, for the presence of *L. lactis*. In particular, one millilitre of milk was submitted to DNA extraction (Cocolin et al., 2001) and the presence of *L. lactis* investigated by using a SYBR Green qPCR protocol, specific for *L. lactis* detection, and optimized by Ruggirello et al. (2014)(described in paragraph "Bulk analysis and qPCR").

Finally, pH values were measured in milk samples immediately after inoculum, curd after draining and cheese at the end of ripening.

Traditional plating and species-specific PCR. Ten millilitres or grams of each sample were homogenised in 40 mL of sterile Ringer solution (Oxoid, Milan, Italy) in a Stomacher (Interscience, Rockland, MA, USA) for 5 min. The resulting suspension was serially diluted and plated on M17 agar

(Biolife, Milan, Italy) supplemented with lactose (5 g/L). The plates were incubated, aerobically, at 30 °C for 48 h and, after counting, 10 randomly selected colonies were isolated for each sample and analysed for their belonging to the species *L. lactis*. A total of 960 isolates were grown overnight at 37 °C in M17 broth (Biolife) supplemented with lactose (5 g/L) and submitted to the DNA extraction according to the protocol described by Cocolin et al. (2001). The identification of *L. lactis* isolates was performed by combining PCR 16S–23S rRNA gene spacer analysis (RSA) (Jensen et al., 1993) and *L. lactis* specific PCR, based on the amplification of a portion of the histidine biosynthesis operon (His-PCR) (Corroler et al., 1998). On the basis of RSA electrophoretic patterns, the isolates showing a single band were checked by His-PCR to confirm their belonging to *L. lactis* species.

Bulk analysis and qPCR

In order to detect, in the inoculated milk, curd and cheese samples, *L. lactis* populations present at low concentration, the matrices diluted 10^{-1} were also plated on M17 agar for bulk formation, described as the continuous patina spread on M17 plates (Ercolini et al., 2001); the surface cell formation was suspended in 1 mL of Ringer, recovered by a sterile L-shape spreader and harvested with a sterile pipette. The cell bulk was stored at –20 °C before DNA extraction (Cocolin et al., 2001). DNA yield and quality were determined with NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and the concentration was standardized, for all the samples, at 200 ng/µL before amplification. In order to investigate the presence of L. *lactis* cells, a SYBR Green qPCR protocol, selective for L. *lactis*, was used (Ruggirello et al., 2014). Briefly, primers Tuf2f (5'-TGA ACC ACA ATG GGT TGC TA-3') and Tuf2r (5'- TCG ACT GGA AGA AGG AGT GG -3') were added at 400 nM and 50 nM, respectively, to the SsoAdvancedTM SYBR@ Green Supermix (Bio-Rad, Hercules, CA, USA) in a final volume of 20 mL. The thermal cycle conditions were as follows: an initial denaturation at 98˚C for 2 min, 40 cycles at 95˚C for 5 sec and 68.7˚C for 30 sec, where the stringent annealing temperature value, and the cumulative annealing and extension steps were chosen to increase the selectivity of the protocol to the microorganism target *L. lactis*.

RT-qPCR assays. Milk, curd and cheese samples, diluted and homogenised as described above, were submitted to direct RNA extraction. One millilitre of the 1:5 diluted suspension of each sample was collected, in duplicate, and centrifuged at 16,100xg for 10 min. The pellets were protected from RNA degradation with RNA later (Ambion, Milan, Italy) and storage at -30°C. RNA extraction was carried out according to the MasterPure™ Complete DNA and RNA Purification Kit (Epicentre, Madison, WI, USA), following the manufacturer's instructions modified uniquely by the addition of lysozyme (50 mg/mL, Sigma, Milan, Italy), at the beginning of the protocol, to improve cell wall lyses. DNase treatment was performed with TURBO-DNase (Ambion) following the manufacturer's instructions. The absence of residual DNA was routinely verified by qPCR using the protocol described above (Ruggirello et al., 2014). Retrotranscription (RT)-qPCR and quantification of alive *L. lactis* cells, were essentially performed as optimized by Ruggirello et al. (2014). Briefly, M-MLV Reverse Transcriptase kit (Promega, Milan, Italy) was used for retrotranscription. RT was performed as follows: 0.5 µg of RNA were mixed with 1 µL of TUF2r primer (100 mM) and 0.6 µL of RNasin ribonuclease inhibitor (20 U/mL) in a reaction volume of 10 µL by addition of ultrapure water. The mix was treated at 75˚C for 5 min for RNA denaturation and immediately placed on ice for 10 min. Five microliters of M-MLV RT Buffer (1X), 5 µL of dNTPs (10 mM each) and 1 µL of M-MLV Reverse Transcriptase (8 U/mL) were added to the mix for a final volume of 25 µL by addition of ultrapure water. RT reaction was carried out at 42˚C for 1 h in a Biorad DNA Engine thermal cycler (Bio-Rad) and the cDNA samples stored at -30˚C. The cDNA was, then, subjected to qPCR, according to the protocol of Ruggirello et al. (2014) and the results obtained in terms of Ct, interpolated in the standard curve equation constructed in a previous study (Ruggirello et al., 2014) according to which quantification limit and detection limit of *L. lactis*, in cheese matrices, were determined, respectively, at 10^2 and 10 CFU/mL or g.

***L. lactis* revitalization from VNC state.** Starter cultures (stK, stY, stB and stJ) which seemed to lose the ability to grow on M17 medium during ripening, as revealed by His-PCR and bulk analysis, were submitted to resuscitation trials. Thus, 180 day ripened cheese samples (Ch180), where *L. lactis* was detected by RT-qPCR but it was not found on M17 medium, were 1:5 diluted and

homogenized, as described above, and inoculated in modified M17 broths. Precisely, 500 µL of the homogenate cheese were inoculated in 10 mL of four broths, added with different percentage of carbohydrates, as follow: M17 broth with lactose at 2% (w/v), and M17 broth with glucose at 0.5%, 1% and 2.5% (all w/v). The broths were, then, incubated at 37 °C and, after 24 h, 100 µL of each suspension were spread on M17 agar plates, supplemented with lactose (5 g/L). After incubation at 30 °C for 48 h, cell bulks were harvested and stored at –20 °C before DNA extraction and qPCR specific for *L. lactis* detection, as described above.

RESULTS AND DISCUSSION

In the next paragraphs, the results about the fate of *L. lactis* in cheese models will be presented in terms of comparison between the two different approaches culture-dependent (plating on M17 medium) and –independent (RT-qPCR specific for *L. lactis*). The data obtained were not always in agreement and this led to the hypothesis of *L. lactis* entering in VNC state during late ripening.

Miniature cheeses and pH measurements

The miniature cheeses weighed, before vacuum packing, on average, 22.50 g ± 0.71. During cheese-making, pH values showed a decrease from 6.6 ± 0.1, in milk after inoculum, to 5.3 ± 0.1 in the curd after draining, and to 4.4 ± 0.1 in cheese at the end of ripening.

The behaviour of the starter cultures, in terms of pH decrease, was comparable with the performance normally expected in Tome Piemontese cheese. The use of cheese models overcomes some of the problems due to the complexity of high-scale productions in terms of manufacture and microbial biodiversity. Thus, it can be considered a valid approach for the screening of a number of starters before scaling up to pilot-scale and standard Tome cheese productions (Shakeel-Ur-R., 1998).

Traditional plating and *L. lactis* detection

By plating on M17 agar, all the starters inoculated were found, after inoculum in the milk, with loads of 10^7-10^8 CFU/mL. The general microbial trend was comparable, during manufacturing, when all the starters reached values of about 10^9 CFU/g, while during ripening they behaved differently. Starters stK, stB, StM kept high loads until the end of ripening with values of 10^8 CFU/g; on the

contrary, starters stW, stY, stJ, stX dropped to 10^5 CFU/g after 90-120 days of ripening and were found with loads ranging from 10^2 CFU/g to 10^5 CFU/g after 180 days of ripening. Finally, stC showed values of 10^7 CFU/g until 150 days of ripening and decreased to 10^4 CFU/g at the end of the 180 days.

Taïbi et al. (2011) used a manufacturing model cheese (Pearce activity test), simulating Cheddar cheese conditions, to follow the performance of four strains of *L. lactis* subsp. *cremoris*.

In that study, the addition of the salt had a negative effect on the growth of the strains and the authors hypothesized that the reduction of viable counts, following osmotic stress, could be attributed to the entering of the cells in a catabolically and/or anabolically inactive state, which made them unable to multiply more than to a process of autolysis. In the present experimentation, the salting step did not cause the arrest of starter growth and the cheesemaking and ripening parameters seemed to affect differently the performance of the eight starters.

The results obtained on M17 medium were further investigated to confirm the presence of colonies on the plates belonging to *L. lactis* species. It is known that cultural media are not always strictly selective towards the declared microbial groups. In particular, it is known that M17 medium shows high efficiency and selectivity when lactococci are abundant and not stressed in the matrix to be analysed, as for example curd and early ripened samples. On the contrary, different authors (Delbès et al., 2007; Ruggirello et al., 2014; Tornadijo et al., 1995) reported a lack of selectivity of M17 medium when, during the ripening process of dairy products, lactobacilli increase in number and prevail on lactococcal populations, which are often out-competed by non-starter lactic acid populations. Thus, 10 colonies for each sampling point were submitted, firstly to RSA-PCR screening. Then, to determine if the counts found on M17 could be totally ascribable to *L. lactis* species, the isolates showing the RSA band migrating approximately 390 bp, characteristic of *L. lactis*, where checked by His-PCR to finally establish their belonging to *L. lactis* species (Dolci et al., 2008).

For two starters, stW and stX, all the colonies analysed belonged to the target species. Thus, even if with low counts, they colonized the cheeses from manufacturing until late ripening (180 days). The starter stM was also found after 180 days of ripening but only 2 of the 10 colonies analysed resulted

to belong to the target species (Table 1). For the other five starters (stK, stY, stB, stJ and stC), a decrease of the number of colonies identified as *L. lactis* was observed from 30 to 120 days of ripening, and in samples ripened for 120, 150 or 180 days they were not found. These data could be interpreted as a lack of M17 selectivity, as reported above, and, in fact, the counts obtained were due to the presence of viable cells belonging to secondary dairy populations. RSA-PCR profiles of these isolates were referable to lactobacilli and, to a lesser extent, to enterococci. In particular, 31 isolates showed a RSA electrophoretic profile characteristic of enterococci (two band migrating approximately at 400 bp and 500 bp or at 350 and 400 bp) while 138 isolates showed three-four RSA bands commonly reported for lactobacilli strains (Dolci et al., 2008).

His-PCR data were also compared with the evaluation of the presence of *L. lactis* directly in cell bulks originated from a sample dilution of 10 to the first, and this approach allowed to analyse the presence of the target cells at a more sensitive level (Tab. 1). For milk, curd and early ripening phases, the data found by His-PCR and cell bulk analysis coincided perfectly. On the contrary, in late ripening, this correspondence was not always respected, since *L. lactis* presence in cell bulks was observed also when no colonies belonged to *L. lactis*, according to His-PCR. Thus, bulk analysis, followed by *L. lactis* specific qPCR, has to be considered a more sensitive and, thus, reliable approach for the detection of culturable cells of the target microorganism on selective medium.

On the basis of cell bulk analysis, the presence of *L. lactis* cultivable cells was detected, for four starters (stW, stC, stM and stX), throughout the whole ripening, including cheese samples at 180 days of ripening. Differently, for the other four starters, at 120, 150 and 180 days of ripening *L. lactis* was not always found, underlining the well-known role of this microorganism as dairy starter and not as secondary culture.

Muehlenkamp-Ulate and Warthesen (1999) studied the performance of *L. lactis* subsp. *cremoris* SK11 in cheese slurries made from curds manufactured under controlled conditions. The slurries simulated accelerated ripening and twelve days were proved to be equivalent to six months of ripening at 4-6°C. Similarly to our results, they found *L. lactis* starter until the end of the process with loads of 10^3 CFU/g. Their explanation was that the high moisture levels of the model cheese and the

high incubation temperature (30 °C) could have aided residual lactose utilization and, thus, *L. lactis* growth.

L. lactis viability by RT-qPCR assays

The RT-qPCR protocol, selective for *L. lactis*, was applied to the total microbial RNA extracted directly from milk, curd and cheese matrices.

The quantification of the alive *L. lactic* populations, obtained by applying the protocol and the standard curves previously described (Ruggirello et al., 2014), is presented in Table 1. From these results, it was clear that the adaptation to milk conditions was different among the starters. Precisely, starters stB, stJ, stC and stX were found in milk with loads of 10^6-10^7 CFU/mL, while starters stK, stW, stY and stM were detected with loads of 10^3 CFU/mL. An explanation to these low values could be found in the quantity of lyophilized cell powder used for the inoculums, according to the producer's instructions, thought for high quantity of milk in vats. The initial small amount of lyophilized powder could be reflected in an initial microbial heterogeneity in terms of both load values and cell physiological state. On the other hand, the discrepancy of the results obtained, for the starters stK, stW, stY and stM, by RT-qPCR and plating on M17 where the counts were higher (and comparable to the ones of starters stB, stJ, stC and stX) (Table 1), can be hardly explained. It could be hypothesized that the permanence in milk at 37°C worked as an enrichment for *L. lactis* but this point remains to be fully clarified.

Independently from their initial inoculum, all the starters reached values of about 10^7-10^8 CFU/g during manufacturing, and their subsequent performance throughout ripening seemed not to be affected from the initial viability found in milk. They showed values ranging from 10^5 to 10^8 CFU/g from 15 to 150 days of ripening, depending on the starter. Noteworthy, *L. lactis* populations were detected in all miniature cheeses, up to the sixth month of ripening, with loads varying from 10 to 10^3 CFU/g, confirming the presence of viable cells of this microorganism during the whole ripening process.

The data obtained by direct RNA analysis were compared with the data observed by traditional plating (Tab. 1). The count values obtained on M17 were generally higher than the ones found by

direct *L. lactis* analysis even if a similar trend was observed during manufacturing and early ripening. The loads found on M17 plates could be interpreted, as already reported, as an overestimation due to the growth of lactobacilli and enterococci along with *L. lactis*. In particular, in late ripening, in a few cheeses, the values reached 10^7-10^8 CFU/g on M17, but no colonies could be related to *L. lactis* by either His-PCR or bulk analysis. As reported above, relative to starters stK, stW, stY and stM, the incongruence of the count values detected in milk samples, by RT-qPCR and traditional plating, can be hardly explained; for sure, microbial RNA extraction from fat matrices has to be considered a weak point and an argument could be if the extraction efficiency from microbial cells in lyophilized state can become an issue.

Differently, RT-qPCR allowed to selectively monitor *L. lactis*, enabling to overcome the issues related to the cultivation step. Noteworthy, *L. lactis* was detected by RT-qPCR in cheese samples (Ch120, Ch150, Ch180 for stK; Ch180 for stY; Ch150, Ch180 for stB; Ch180 for stJ) where it was not found by traditional plating and either His-PCR or cell bulk analysis. This discrepancy could be explain with the fact that lactococci, during ripening process, enter in a stressed physiological state, which might explain the absence of growth on M17 medium despite their viability in cheese matrix. These data underline the usefulness of direct RNA analysis in assessing the viability of *L. lactis,* which could synthesize RNA, although losing the capability to form colonies on synthetic medium. On the other hand, culture-independent methods could show limitations due to the quality of the target molecule. Specifically, purity and integrity of RNA are critical elements for the overall success of RNA-based analyses (Fleige and Pfaffl, 2006) and getting good quality nucleic acids can be tough and laborious when the matrix is complex like cheese. Therefore, several authors suggested that using a polyphasic approach, combining culture-dependent and -independent methods, may be worthwhile to obtain a more accurate view of the structure of microbial communities (Delbès et al., 2007; Ercolini et al., 2001; Flórez and Mayo, 2006).

Our results are in agreement with the studies of Desfossés-Foucault et al. (2013) who evaluated the impact of milk heat treatments and ripening temperatures on lactococcal starter and non starter LAB throughout maturing of Cheddar cheese. According to their data, lactococci retained transcriptional activity for six month of ripening. The same authors (Desfossés-Foucault et al., 2014) analysed the

transcriptional profile of two lactic strains, *L. lactis* subsp. *cremoris* SK11 and *Lactobacillus paracasei* ATCC 334, in mixed culture, during simulation of Cheddar cheesemaking (Pearce activity test) and ripening (slurries). They detected cDNA transcripts until the end of ripening, while *L. lactis* SK11 populations rapidly declined to totally disappear, in early ripening stages, on M17 medium.

Recent studies highlighted the viability of lactococcal populations in ripened commercial and artisanal cheeses. Dolci et al. (2014) monitored *L. lactis*, by RT-PCR–DGGE and pyrosequencing, in Fontina PDO cheese, where it was inoculated as starter together with *Streptococcus thermophilus* and *Lb. delbrueckii*, and it was found up to three months of ripening. Similarly, other authors detected the microorganism, by culture-independent methods, in Danish cheeses (Masoud et al., 2011), Castelmagno PDO (Dolci et al., 2010), Feta PDO (Rantsiou et al., 2008), Toma PDO, Raschera PDO, Asiago PDO and Pecorino sardo PDO (Ruggirello et al., 2014).

Sheehan et al.(2007) described that *L. lactis* subsp. *lactis* survives better in cheese than *L. lactis* subsp. *cremoris*. This assumption was also suggested by Gori et al., (2013) who investigated the microbiota of different Danish farmhouses surface-ripened cheeses by both dependent and independent approaches. *L. lactis* subsp. *cremoris* was generally not found in the cheeses at the end of ripening, even though it was added as a part of the primary LAB starter culture. On the contrary, we did not observe any difference in the performance of the two subspecies in terms of persistence during cheese aging.

Lastly, the comparison of the results obtained, in the present study, by both culture-dependent and -independent analysis suggested, as reported by other authors (Chou et al., 2001; Ganesan and Weimer, 2004; Ganesan et al., 2007; Stuart et al., 1999), that *L. lactis* entered in a VNCS during cheese aging. Sugar starvation has been recognized as a key stress for LAB, especially lactococci, although also others stress factors, as pH or temperature, can contribute to VNCS, during fermentation process (Weimer, 2011). Some authors (Ganesan et al.,2007; Stuart et al., 1999) demonstrated that carbohydrate starvation leads *L. lactis* in VNCS within 12-48 h. During this time, the microorganism shifts its metabolism from glycolysis to amino acid catabolism, playing an important role in cheese flavour. During the transition to the VNCS, the cells lose the ability to divide,

appearing to decrease in number or even die, by classical plating, but remaining intact and metabolically active to produce secondary metabolites (Ganesan et al., 2006).

Noteworthy, among the starters used in the present study, we observed a strain-dependence attitude, concerning the moment of entering in VNCS. This could be associated at the different ability of *L. lactis* to exhaust lactose, which delayed the entry in this state, as already demonstrated by Stuart et al. (1999).

L. lactis revitalization

The VNC state in bacteria has always been a complex and controversial topic. Sceptics for its existence were largely driven by the inability to wake up the cells after they became VNC (Weimer, 2011). The resuscitation of VNC cells has been possible for some Gram-negative *Proteobacteria* and a few *Actinobacteria* but it is still to be demonstrated in LAB.

In order to support the hypothesis that the commercial starters studied here entered in VNCS, during late ripening, a trial to resuscitate *L. lactis* cells was performed.

According to some authors (Ganesan et al., 2007; Stuart et al., 1999), carbon sources seem to affect the culturable state of *L. lactis*; in particular, carbohydrate-starved lactococci attain a nonculturable state wherein sugar metabolism, cell division, and autolysis are repressed; in parallel, cells maintain transcription, metabolic activity, and energy production during a state thatproduces new metabolites not associated with growth. Thus, on the basis of these evidences, different carbohydrate, at different percentages (lactose at 2% (w/v) and glucose at 0.5%, 1% and 2.5% (w/v), added at M17 medium), were chosen, in our study, in order to stimulate the cells to become culturable again.

The results obtained by bulk cell analysis, highlighted that the enrichment step affected, in a strain-dependent way, the ability of *L. lactis* starters to grow again on synthetic medium. Precisely, 2.5% glucose addition was effective to recover the cells of the starters stB and stY from the VNC state while starters stK and stJ were not revitalized by any of the diverse nutritional conditions tested.

As reported above, carbohydrates play a critical role in the viability of lactococci and the formulation of specific nutritional media should be better investigated for a more effective recovery of stressed microbial cells.

CONCLUSIONS

In the present study, the use of miniature cheeses allowed to follow different *L. lactis* starters throughout manufacturing and ripening, and new insights were reached on the capability of this microorganism to persist during the whole process and up to six months of aging. In particular, *L. lactis* showed a strain dependent behaviour, especially in the last months of cheese ripening, when it entered in a hypothetical VNCS. Preliminary results obtained by "resuscitation" assays corroborated this observation, but additional studies will be needed to demonstrate and clarify the events associated with this physiological state.

The capability of *L. lactis* to persist, in late ripening, suggests a possible technological role of this microorganism in cheese. It has been demonstrated that during sugar starvation, in ripened products, *L. lactis* VNC cells switch from glycolysis to nitrogen catabolism. Hence, even if the microorganism appears to die because it cannot form colonies on synthetic medium, yet the cells are intact and continue to metabolize peptides and amino acids to end products that could affect flavour. Even if the biological significance of the VNCS remains to be demonstrated, the impact of this microbial metabolic state cannot be ignore in fermented food, such as cheeses. Additional efforts are needed to better interpret *L. lactis* VNCS in cheese and to investigate its contribution to flavour formation in final products; moreover, as future prospective, it will be important to support these data with pilot and industrial scale studies.

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Table 1. Detection of alive cells of *L. lactis* throughout manufacturing and ripening of miniature cheeses: comparison of the results obtained by culture-independent and -dependent approaches.

	STY				STB				STJ			
Sample[a]	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk[c]**	**His[d]**	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**
	Mean[b] ± SD	Mean ± SD			Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD		
M			+	10			+	10			+	10
			+	10			+	10			+	10
CuC			+	10			+	10			+	10
			+	10			+	10			+	10
CuD			+	10			+	10			+	10
			+	10			+	10			+	10
ChS	3.87 ± 0.15	7.60 ± 0.45	+	10	6.99 ± 0.71	8.01 ± 0.59	+	7	5.91 ± 0.57	8.06 ± 0.63	+	8
	4.98 ± 0.17	8.59 ± 0.04	+	9	7.87 ± 0.10	8.82 ± 0.35	+	5	6.99 ± 0.23	9.38 ± 0.50	+	8
Ch7	5.34 ± 0.21	9.41 ± 0.23	+	9	7.99 ± 0.08	9.46 ± 0.16	+	5	7.12 ± 0.44	9.78 ± 0.57	+	6
	6.70 ± 0.90	10.60 ± 0.71	+	9	8.24 ± 0.74	9.69 ± 0.23	+	0	6.40 ± 0.54	9.95 ± 0.54	+	5
Ch15	7.00 ± 0.56	8.46 ± 0.52	+	8	7.27 ± 0.62	9.18 ± 0.42	-	0	5.99 ± 0.87	9.29 ± 0.54	+	0
	6.98 ± 0.04	8.45 ± 0.15			6.33 ± 0.32	9.02 ± 0.45			5.70 ± 0.01	9.04 ± 0.65		
Ch30	6.78 ± 0.11	7.78 ± 0.47			4.96 ± 0.37	8.75 ± 0.22			5.65 ± 0.21	7.70 ± 0.60		
	4.55 ± 0.28	7.00 ± 0.42			4.81 ± 0.44	8.66 ± 0.11			5.42 ± 0.96	6.30 ± 0.59		
Ch60	3.07 ± 0.33	7.41 ± 0.06			4.81 ± 0.44	8.48 ± 0.01			4.99 ± 0.01	4.48 ± 0.54		
	2.39 ± 0.72	5.78 ± 0.17			4.75 ± 0.46	8.81 ± 0.57			4.58 ± 0.28	4.50 ± 0.02		
Ch90	1.44 ± 0.46	3.90 ± 0.92			4.67 ± 0.19	7.90 ± 0.28			3.41 ± 0.27	4.15 ± 0.08		
	1.40 ± 0.28	4.08 ± 0.62	-	0	3.81 ± 0.35	7.76 ± 0.43	-	0	3.38 ± 0.69	5.95 ± 0.88	-	0
Ch120												
Ch150												
Ch180												

	StK				StW				StM			
	RNA (CFU/g)	**M17 (LOG CFU/g)**	**Bulk**	**His**	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**
	Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD		
M	3.27 ± 0.77	7.70 ± 0.73	+	10	4.29 ±0.13	8.40 ± 0.63	+	10	3.20 ± 0.42	8.57 ± 0.97	+	10
	4.30 ± 0.65	9.27 ± 0.32	+	10	6.79 ± 0.04	8.93 ± 0.21	+	10	4.45 ± 0.21	8.99 ± 0.22	+	10
	6.80 ± 0.66	9.61 ± 0.54	+	10	7.23 ± 0.20	9.46 ± 0.77	+	10	8.17 ± 0.19	9.45 ±0.91	+	10

CuC	6.91 ± 0.47	9.52 ± 0.34	+	10	7.85 ±0.78	9.77 ± 0.25	+	10	8.23 ± 0.11	9.43 ± 0.10	+	10
	7.05 ± 0.17	9.20 ± 0.67	+	10	4.46 ± 0.04	9.52 ± 0.21	+	10	8.00 ± 0.04	9.33 ± 0.14	+	10
CuD	7.19 ± 0.65	8.26 ± 0.23	+	10	4.42 ± 0.19	8.41 ± 0.03	+	10	7.99 ± 0.23	8.48 ± 0.04	+	10
	4.09 ± 0.49	8.45 ± 0.53	+	8	4.35 ± 0.58	8.28 ± 0.39	+	10	7.54 ± 0.54	7.66 ± 0.59	+	10
ChS	3.26 ± 0.79	8.15 ± 0.15	+	6	3.90 ± 0.78	7.95 ± 0.08	+	10	7.41 ± 0.04	7.49 ± 0.05	+	10
	2.76 ± 0.55	7.48 ± 0.24	+	6	3.78 ± 0.37	6.00 ± 0.23	+	10	6.98 ± 0.05	8.51 ± 0.19	+	10
Ch7	2.71 ± 0.47	8.25 ± 0.39	-	0	3.54 ± 0.76	5.00 0.04	+	10	6.04 ± 0.04	8.60 ± 0.08	+	10
	2.63 ± 0.94	8.68 ± 0.62	-	0	3.41 ± 0.12	4.90 ± 0.17	+	10	4.64 ± 0.02	7.20 ± 0.31	+	9
Ch15	2.60 ± 0.34	8.20 ± 0.21	-	0	2.55 ± 0.02	2.90 ± 0.17	+	10	2.80 ± 0.63	8.40 ± 0.49	+	2
Ch30												
Ch60												
Ch90												
Ch120												
Ch150												
Ch180												

Table 1 (continued)

	StX				**StC**			
Sample	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**	**RNA (CFU/g)**	**M17 (LOG CFU/g)**	**Bulk**	**His**
	Mean ± SD	Mean ± SD			Mean ± SD	Mean ± SD		
M	6.13 ± 0.06	6.70 ± 0.02	+	10	6.14 ± 0.14	7.85 ± 0.31	+	10
CuC	7.24 ± 0.04	7.90 ± 0.79	+	10	6.37 ± 0.34	8.66 ± 0.23	+	10
CuD	7.76 ± 0.46	9.13 ± 0.04	+	10	6.52 ± 0.04	9.51 ± 0.43	+	10
ChS	6.40 ± 0.63	8.72 ± 0.83	+	10	7.00 ± 0.59	9.78 ± 0.52	+	10
Ch7	5.28 ± 0.40	8.18 ± 0.67	+	10	7.08 ± 0.07	9.43 ± 0.41	+	10
Ch15	3.92 ± 0.54	8.00 ± 0.01	+	10	7.87 ± 0.07	9.47 ± 0.46	+	10
Ch30	3.33 ± 0.23	7.63 ± 0.39	+	10	8.15 ± 0.12	8.93 ± 0.39	+	10

Ch60	3.27 ± 0.21	7.00 ± 0.23	+	10	6.78 ± 0.15	7.57 ± 0.50	+	10
Ch90	3.25 ± 0.13	7.56 ± 0.13	+	10	5.37 ± 0.32	7.85 ± 0.26	+	7
Ch120	2.73 ± 0.13	4.90 ± 0.17	+	10	2.52 ± 0.59	7.51 ± 0.28	+	4
Ch150	2.65 ± 0.27	3.95 ± 0.30	+	10	2.31 ± 0.47	7.00 ± 0.11	+	0
Ch180	2.44 ± 0.56	2.36 ± 0.81	+	10	1.71 ± 0.34	4.30 ± 0.41	+	0

[a] M. milk after inoculum of the starter (the loads were expressed as CFU/ml); CuC. curd after cutting; CuD. curd after drying; ChS. cheese after salt; Ch7. 15. 30. 60. 90. 120. 150. 180: cheese after 7. 15. 30. 60. 90. 120. 150. 180 days of ripening.

[b] The values, expressed as microbial loads referred to standard curve described in a previous work (Ruggirello et al. 2014).

[c] "+": *L. lactis* was detected; "-": *L. lactis* was absent.

[d] Referred to His-PCR: number of isolates found belonging to *L. lactis* species on a total of 10 colonies analysed.