Lactococcus lactis viability in cheese ripening

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Introduction

Recent studies, based on culture-independent analysis, have shown the presence of *Lactococcus lactis* in ripened dairy products (Desfossés-Foucault et al., 2013; Flòrez and Mayo, 2006; Masoud et al., 2011, Ruggirello et al., 2014). On the other hand, traditionally methods based on cultivation of lactic acid bacteria (LAB) on selective media rarely highlighted the presence of this species 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 viable but not cultivable state (VNCS), which is a current complex and controversial topic. Up to now, the molecular events associated with the VNCS are not yet well delineated and no direct approaches to detect and resuscitate metabolically active cells of *L. lactis* are available to directly prove the existence of this state. The aim of our study was to confirm the presence of viable *L. lactis* cells in late ripened commercial cheeses (1); to monitor the persistence of *L. lactis* in model cheeses, and evaluate the time of its entry in VNCS (2); to revitalize *L. lactis* VNC cells by enrichment in modified media (3).

(1) Detection of *L. lactis* in ripened commercial cheeses

Materials and Methods

a. Optimisation of a selective RT-qPCR protocol to detect *L. lactis* in cheese matrices.
b. Analysis of 33 ripened cheeses, available on the market, by a double approach:
   1. Culture-independent analysis: 10 g of each miniature cheese were homogenized with 40 mL of Ringer solution and an aliquot (1 mL) was submitted to the detection of the presence and viability of *L. lactis* by RT-qPCR.
   2. Culture-dependent analysis: the cheese samples were analysed by traditional plating on lactococci selective medium M17 agar. Ten colonies, for each sample, were isolated and submitted to DNA extraction and *L. lactis* species-specific PCR.

Results

L. lactis was found, by RT-qPCR direct analysis, in 25 samples of the following cheese: Asiago PDO, Castelmagno, Fontina PDO, Pecorino Fioretto, Pecorino Fiore Sardo, Pecorino Toscano PDO, Raschera PDO, Toma Piemontese PDO, Toma di Lanzo. The loads found on plates were ranging from 10^3 to 10^8 CFU/g but only in 8 cheese samples, the colonies were positive to species-specific PCR and, thus, resulted belonging to *L. lactis*.

The absence or low abundance of *L. lactis* colonies on M17 medium (in samples where it was found by RT-qPCR) supported the thesis of the presence *L. lactis* cells in VNCS. Thus, to better asses the behaviour of this microorganism throughout cheese-making and ripening, a model cheese was used (2).

(2) Persistence and viability of *L. lactis* in model cheeses

Materials and Methods

a. Manufacturing of miniature cheeses according to the protocol of Shakeel-Ur-Rehman et al. (1998) with 8 commercial starters (*L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris*).
b. Ripening of miniature cheeses at 8°C vacuum for 6 months.
c. Sampling of miniature cheeses 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).
d. Analysis by culture-independent (RT-qPCR) and dependent methods. In addition to microbial counts and species -specific PCR, the plates obtained from 10^{-1} diluted cheese samples were used for bulk formation (Ercolini et al., 2001). One millilitre of bulk cell suspension was collected and submitted to DNA extraction and qPCR for the detection of *L. lactis.*

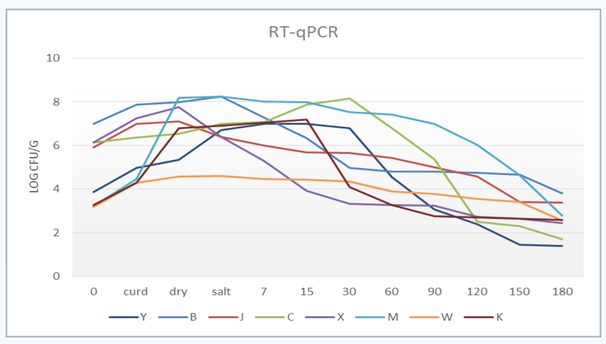

Fig. 1. Quantification by RT-qPCR of metabolically active cells of *L. lactis* starters throughout manufacturing and ripening of miniature cheeses.

* B, J, M, K, C: *L. lactis* subsp. *lactis* starters; X and Y: *L. lactis* subsp. *cremoris* starters; W: mix of *L. lactis* subsp. *lactis* and *cremoris*.

Results

L. lactis populations were detected by direct RNA analysis (RT-qPCR) in all miniature cheeses, up to sixth month of ripening when the loads were varying from 10 to 10^3 CFU/g (Fig. 1), confirming the presence of viable cells of this microorganism during the whole ripening process. On the contrary, in late ripening, the presence of *L. lactis*, was confirmed, by traditional plating, only for 4 starters.
These evidences suggest that *L. lactis* was entering in VNCS during cheese aging. Moreover, we observed a strain-dependence attitude, concerning the time of entering in this state; this could be associated with the different ability of *L. lactis* to exhaust lactose, which could delay the entry in this state, as already demonstrated by Stuart et al. (1999).

In order to corroborate the hypothesis that the commercial starters studied entered in VNCS, during late ripening, a trial to resuscitate *L. lactis* cells was performed (3).

(3) *L. lactis* revitalization

Tab. 1: Trial of revitalization of *L. lactis* in VNCS.

Cheese sample at 180 ripening days	Growth in M17 after supplement of different carbon sources			
	lac 2%	glu 0.5%	glu 1%	glu 2.5%
Y	-	-	-	+
J	-	-	-	-
K	-	-	-	+
B	-	-	-	-

"+", *L. lactis* was detected; "-", *L. lactis* was not detected

Materials and Methods

a. Incubation of 10 grams of 180 day ripened cheese samples (where L. lactis was detected by RT-qPCR but it was not found on M17 medium) in modified M17 broths with different percentage of carbon source (M17 broth with lactose at 2%, M17 broth with glucose at 0,5 %, 1% or 2,5 %) at 37°C for 24h. Plating of 100 µL of each cheese sample suspension on M17 agar plates, supplemented with lactose (5g/L) and incubation at 30°C for 48h.
b. Bulk analysis, as described above.

Preliminary results.
The enrichment step affected, in a strain-dependent way, the ability of L. lactis starters, previously found not culturable, to grow again on synthetic medium. Precisely, 2.5% glucose addition was effective to recover the cells of the starters Y and K while the other two starters, J and B, were not revitalized by any of the diverse nutritional conditions tested.

Conclusions

During sugar starvation, in ripened products, it has been demonstrated that *L. lactis* VNC cells switch from glycolysis to nitrogen catabolism. Even if the biological significance of VNCS remains to be demonstrated, the impact of this microbial metabolic state cannot be ignore in fermented food, such as cheeses. In fact, the capability of *L. lactis* to persist, in late cheese ripening, could suggest a possible technological role of this microorganism in cheese. Additional efforts are needed to better interpret *L. lactis* VNCS in cheese and to investigate its contribution to flavour formation in final products; the formulation of specific nutritional media should be better investigated for a more effective recovery of stressed microbial cells. Furthermore, as future prospective, it will be important to support these data with pilot and industrial scale studies.

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