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Detection and viability of *Lactococcus lactis* during ripening of model cheeses

Marianna Ruggirello*, Luca Cocolin, Paola Dolci,

Department of Agricultural, Forest and Food Sciences, University of Turin, Italy

***email: marianna.ruggirello@unito.it**

Aim of the work

Recent studies (Rantsiou et al. 2008, Dolci et al. 2010) have shown the presence, in ripened cheeses, of alive cells of *L. lactis* by using culture-independent techniques. For this reason, the role of this microorganism, well known as dairy starter, should be reconsidered throughout cheese manufacturing and ripening. It should be considered the possibility that starter populations are in a viable but not culturable (VNC) state during cheese ripening and, for this reasons, culture-dependent methods are not able to highlight their presence. In support of this hypothesis, VNC state has been described for *L. lactis* as a physiological response to carbohydrate starvation (Ganesan et al. 2007), a situation that characterizes a cheese after fermentation (Stuart et al. 1999). These considerations impose a more careful understanding of the role of *L. lactis*, in the ripening process, not only in terms of autolytic activity but also as metabolically active cells.

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. At the same time, culture-dependent and –independent approaches were compared in order to investigate the possible presence of VNC cells of *L. lactis* in cheese.

Materials and Methods

Table 1. Starter cultures of *L. lactis* inoculated in pasteurized milk to manufacture the miniature cheeses.

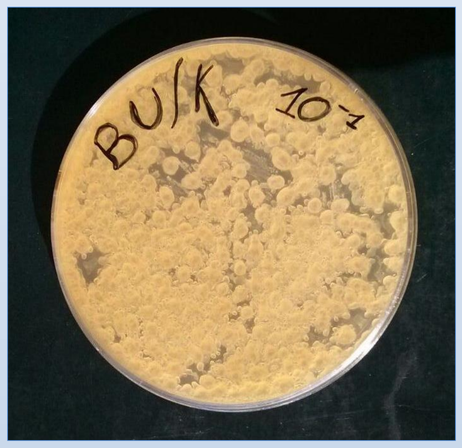
STARTER CULTURES	CODE
L. lactis subsp. lactis	B
L. lactis subsp. *lactis*	J
L. lactis subsp. *lactis*	M
L. lactis subsp. *lactis*	K
Mixture *L. lactis* subsp. *lactis* and *cremoris*	W
L. lactis subsp. *cremoris*	X
L. lactis subsp. lactis	C
L. lactis subsp. *Cremoris*	Y

1. **Manufacturing of miniature cheeses according to the protocol of Shakeel-Ur-Rehman et al. (1998) with eight commercial starters (Table 1).**
2. **Ripening of miniature cheeses at 8°C vacuum for 6 months.**
3. **Sampling 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).**
4. **<u>Culture-independent analysis</u>: 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 protocol optimized in a previous work (Ruggirello et al. 2013).**

5. **<u>Culture-dependent analysis</u>: the cheese samples were analysed by traditional plating on lactococci selective medium M17 agar.**

5.1 **Ten colonies, for each sample analysed, were isolated and submitted to DNA extraction and *L. lactis* species-specific PCR (His-PCR).**

5.2 **The plates obtained from the cheese sample 10^{-1} dilution 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.***

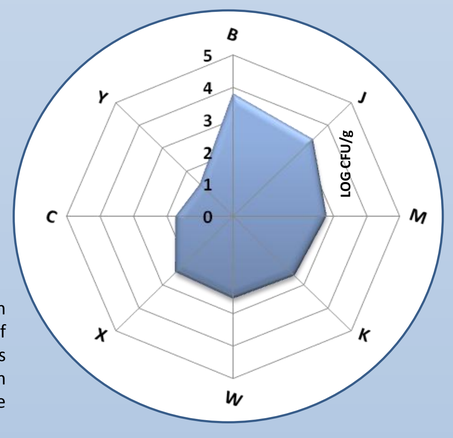


Results

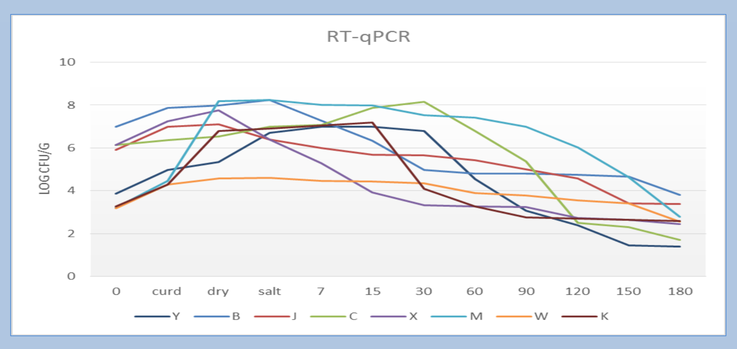
Table 2a, 2b. Detection of alive cells of *L. lactis* throughout manufacturing and ripening of miniature cheeses: comparison of the results obtained by culture-independent and -dependent approaches.

starters		Y				B				J				C			
	Step	RNA analysis (CFU/g)*	M17 Log CFU/g	BULK **	HIS-PCR ***	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR
Manufacturing	milk	3,87	7,60	+	10	6,99	8,01	+	10	5,91	8,06	+	10	6,14	7,85	+	10
	curd	4,98	8,59	+	10	7,87	8,82	+	10	6,99	9,38	+	10	6,37	8,66	+	10
	dry	5,34	9,41	+	10	7,99	9,46	+	10	7,12	9,78	+	10	6,52	9,51	+	10
	salt	6,70	10,60	+	10	8,24	9,69	+	10	6,40	9,95	+	10	7,00	9,78	+	10
Ripening	7d	7,00	8,46	+	10	7,27	9,18	+	10	5,99	9,29	+	10	7,08	9,43	+	10
	15d	6,98	8,45	+	10	6,33	9,02	+	10	5,70	9,04	+	10	7,87	9,47	+	10
	30d	6,78	7,78	+	10	4,96	8,75	+	7	5,65	7,70	+	8	8,15	8,93	+	10
	60d	4,55	7,00	+	9	4,81	8,66	+	5	5,42	6,30	+	8	6,78	7,57	+	10
	90d	3,07	7,41	+	9	4,81	8,48	+	5	4,99	4,48	+	6	5,37	5,85	+	7
	120d	2,39	5,78	+	9	4,75	8,81	+	0	4,58	<1000	+	0	2,52	7,51	+	4
	150d	1,44	3,90	+	8	4,67	7,90	-	0	3,41	4,15	+	0	2,31	7,00	+	0
	180d	1,40	4,08	-	0	3,81	7,76	-	0	3,38	5,95	-	0	1,71	4,30	+	0

*The values, expressed as microbial loads, referred to standard curve described in a previous work (Ruggirello et al. 2013)
** "+": *L. lactis* was detected; "-": *L. lactis* was not detected.
*** Number of isolates found belonging to *L. lactis* species on a total of 10 colonies.



Graphic 1. Quantification by RT-qPCR of metabolically active cells of *L. lactis* starters at sixth month of miniature cheese ripening.



Graphic 2. Quantification by RT-qPCR of metabolically active cells of *L. lactis* starters throughout manufacturing and ripening of miniature cheeses.

Culture-independent analysis

On the basis of RNA analysis, *L. lactis* was found with loads varying from 10^4 CFU/g to 10^8 CFU/g during manufacturing process, and from 10^1 CFU/g to 10^8 CFU/g in ripened samples. Noteworthy, *L. lactis* was found in all miniature cheeses until the sixth month of ripening with loads up to 10^3 CFU/g.

Culture-dependent analysis

Traditional plating on M17 medium led to loads ranging from 10^2 to 10^{10} CFU/g, including cheese samples were no *L. lactis* was found by His-PCR on colonies and qPCR applied to bulk. These data could be interpreted as a lack of selectivity of M17 medium where colony growth is not always related to lactococcal species. According to bulk results, four *L. lactis* starters (C, W, M, X) were viable and culturable until the sixth month of ripening; conversely, the starters Y, B, J and K were not found on M17 plates obtained from ripened cheese samples.

His-PCR partially confirmed the results obtained from bulk analysis except for the samples C_{150d}, C_{180d}, J_{120d}, J_{150d}, B_{120d} where *L. lactis* was not found by species-specific PCR on the colonies isolated.

Thus, bulk analysis followed by qPCR showed a higher level of detection of *L. lactis* and, thus, it can be considered a more reliable approach.

Table 2b.

starters		K				W				M				X			
	Step	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR	RNA (CFU/g)	Plate counts Log CFU/g	BULK	HIS-PCR
Manufacturing	milk	3,27	7,70	+	10	3,17	8,40	+	10	3,20	8,57	+	10	6,13	6,70	+	10
	curd	4,30	9,27	+	10	4,29	8,93	+	10	4,45	8,99	+	10	7,24	7,90	+	10
	dry	6,80	9,61	+	10	4,57	9,46	+	10	8,17	9,45	+	10	7,76	9,13	+	10
	salt	6,91	9,52	+	10	4,60	9,77	+	10	8,23	9,43	+	10	6,40	8,72	+	10
Ripening	7d	7,05	9,20	+	10	4,46	9,52	+	10	8,00	9,33	+	10	5,28	8,18	+	10
	15d	7,19	8,26	+	10	4,42	8,41	+	10	7,99	8,48	+	10	3,92	8,00	+	10
	30d	4,09	8,45	+	8	4,35	8,28	+	10	7,54	7,66	+	10	3,33	7,63	+	10
	60d	3,26	8,15	+	6	3,90	7,95	+	10	7,41	7,49	+	10	3,27	7,00	+	10
	90d	2,76	7,48	+	6	3,78	6,00	+	10	6,98	8,51	+	10	3,25	7,56	+	10
	120d	2,71	8,25	-	0	3,54	5,00	+	10	6,04	8,60	+	10	2,73	4,90	+	10
	150d	2,63	8,68	-	0	3,41	4,90	+	10	4,646	7,20	+	9	2,65	3,95	+	10
	180d	2,60	8,20	-	0	2,55	2,90	+	10	2,80	8,40	+	2	2,44	2,36	+	10

Conclusions

This study highlighted the capability of *L. lactis* to persist, as metabolically active cells, throughout manufacturing and ripening in model cheeses. In fact, the starter cultures used in this work were detected by RT-qPCR until the six month of ripening, confirming their presence and viability during the process.

As future prospective, it will be important to investigate the role of *L. lactis*, in terms of metabolic activities, during cheese ripening. In particular, it will be interesting to understand which *L. lactis* functions are being carried out in each specific phase of the production and maturing, with the final aim of improving technological processes and cheese quality.

The data obtained by culture-dependent and –independent analysis were compared and they did not always reach the same results. In fact, in late ripening, the presence of *L. lactis*, detected by RT-qPCR, was not always confirmed by traditional plating. It seems that lactococci, during ripening process, enter in a stressed physiological state which might explain the absence of growth on M17 medium. Moreover, this VNC state attitude seems to be strain-dependent, considering the different behaviour of the starters used in this work. In order to overcome biases associated to the culturing step, direct analysis of microbial RNA proved to be fundamental and able to give a more realistic "picture" of cheese microbiota. As future prospective, for a more reliable and effective recovery of lactococci in stressed state, the optimization and formulation of specific nutritional media should be better investigated.

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