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# Vitality of *Lactococcus lactis* throughout cheese ripening

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## Aim of the work

Quantitative PCR (qPCR) and retrotranscription-qPCR (RT-qPCR) assays were optimized to detect, quantify and determine the vitality of *Lactococcus lactis* in ripened cheeses, where it is known that no starter lactic acid bacteria prevail on lactococcal microbiota. The new protocols were used to screen the presence and vitality of *L. lactis* in commercial cheeses characterized by different ripening times.

Traditional culture-dependent analysis were also carried out in order to evaluate the presence of metabolically active *L. lactis* cells in a viable but not culturable (VNC) state. In previous researches, a few authors detected the presence of this microorganism throughout cheese ripening and hypothesized its contribution to the characteristics of the final product. In the present work we are considering these preliminary evidences.

## Products

| PRODUCTS                         | COD.      |
|----------------------------------|-----------|
| ASIAGO PDO 40 GG                 | ADF40     |
| FONTINA PDO 180 GG               | FD180     |
| PECORINO FIORETTO 140 GG         | PF4-5     |
| RASCHERA PDO CISPINO             | RDC       |
| TOMA PIEMONTESE PDO PEZZANA      | TPDP      |
| PECORINO SARDO DOLCE PDO         | PSDD      |
| TOMA DI LANZO                    | TL        |
| PEC. TOSCANO PDO STAG. 90/180 GG | PTD3-6    |
| FONTINA AOSTA PDO 90 GG CPFA     | FAD90     |
| CASTELMAGNO PDO                  | CDT       |
| BRA PDO                          | BTD       |
| ASIAGO D'ALLEVO PDO 90/120 GG    | AAD90-120 |

## 1. Optimisation of qPCR and RT-PCR protocols

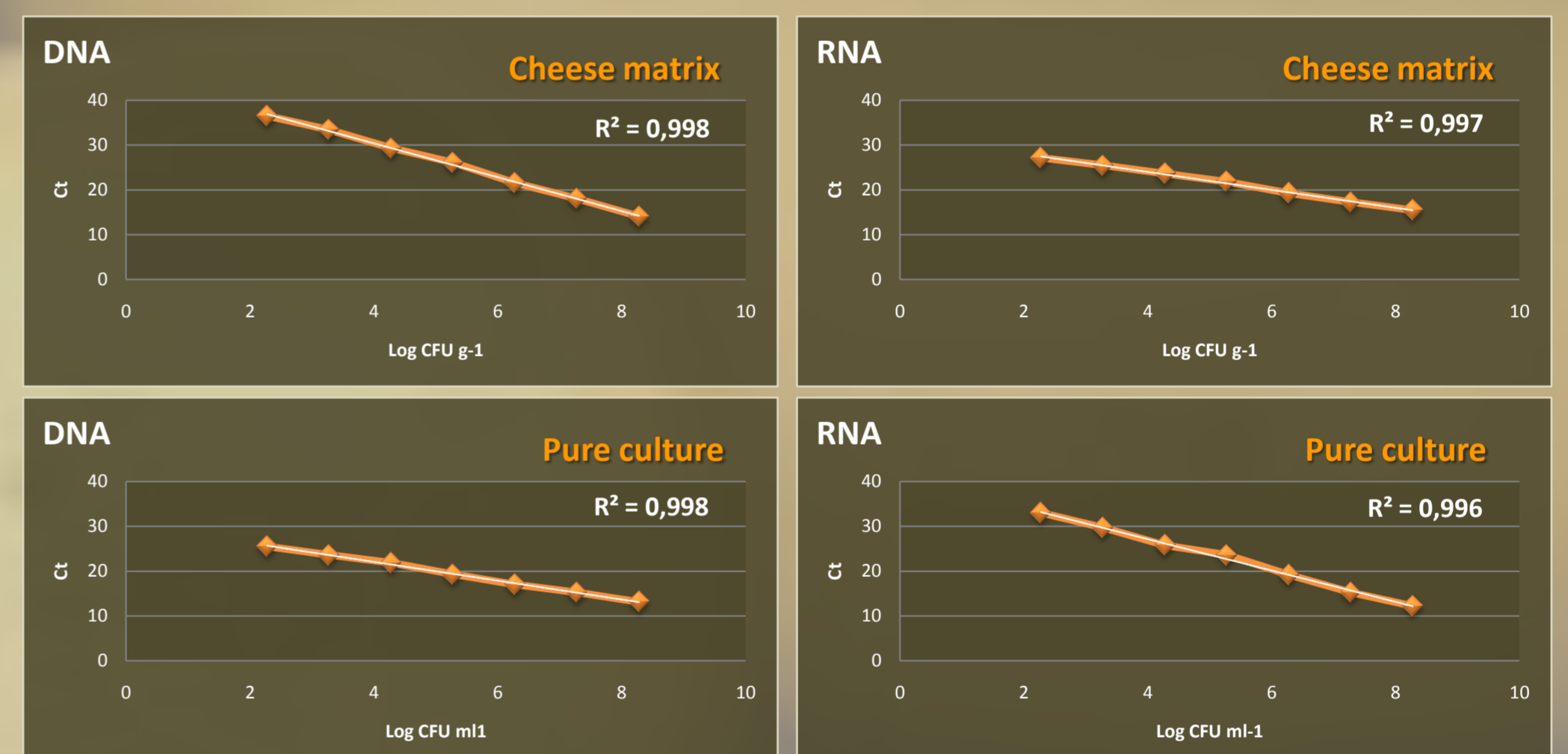
| Amplification conditions                               | Amplification Cycle   |
|--|-----------------------|
| SsoAdvanced™<br>SYBR® Green<br>Supermix 1X<br>(Biorad) | 98°C 2'               |
| Tuf2 f 0,4 µM<br>Tuf2 r 0,05 µM                        | 95°C 5"<br>68.7°C 30" |
|  | 40 cycles             |

The primers Tuf2 were unbalanced (Fig. 1) and different annealing temperatures were tested to optimize the PCR conditions. The protocols were specific for *L. lactis* and did not amplify the other dairy lactic acid bacteria species considered.

Fig. 1. qPCR and RT-qPCR protocols

## 2. Standard Curves

Calibration curves were constructed from serially diluted cells of *L. lactis* as pure culture and inoculated in fresh grated cheese used as biological model. The signals produced in terms of Ct by the decimal serial dilutions were plotted against the Log<sub>10</sub> CFU and standard curves were defined for both DNA and RNA analysis with a quantification limit of 10<sup>2</sup> CFU/g (in cheese).



## 4. Microbiological analysis

The cheese samples resulted positive for the presence of *L. lactis* populations by RT-qPCR, were analysed by traditional plating on lactococci selective medium M17 agar. Twelve colonies for each sample were submitted to DNA extraction and *L. lactis* species-specific PCR (His-PCR) was performed.

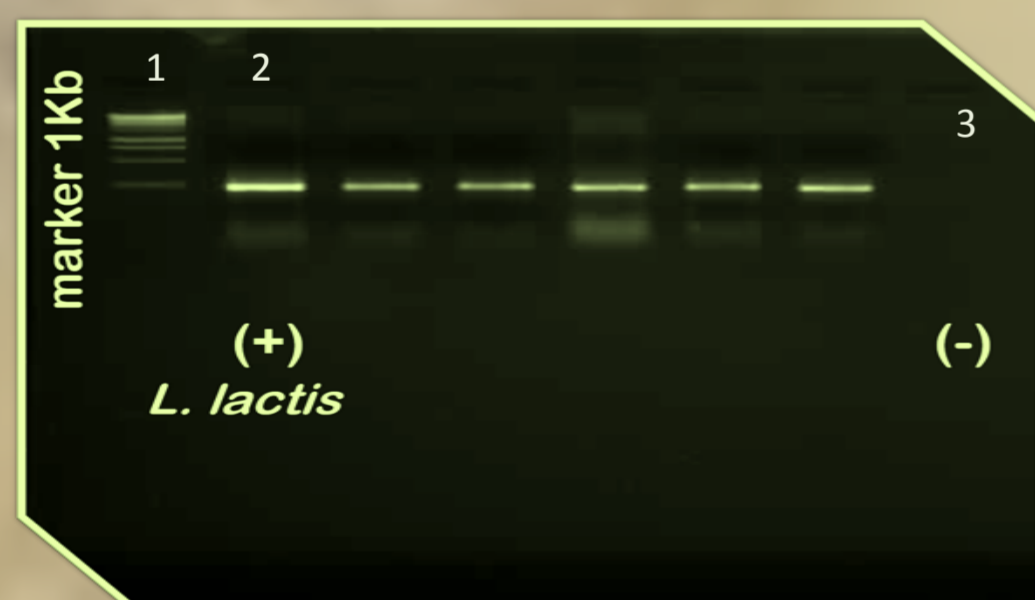
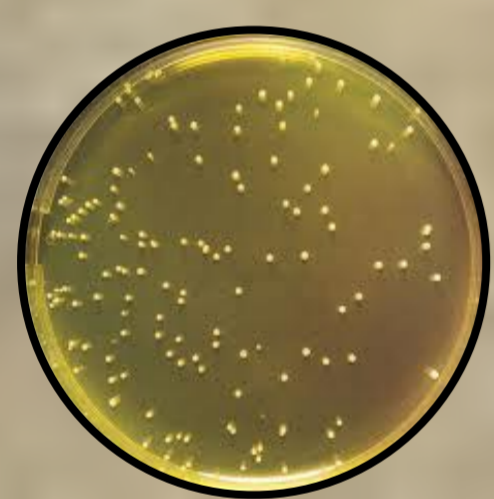


Figure 2. His-PCR profile of *L. lactis* species (+). Line 1: marker; line 2: positive control; line 3: negative control.

## 5. HIS - PCR

His-PCR was carried out with primers HIS I and HIS II, amplifying the operon for histidine biosynthesis in *L. lactis*. The resulting band of 933 bp specific for *L. lactis* is showed in Fig. 2.

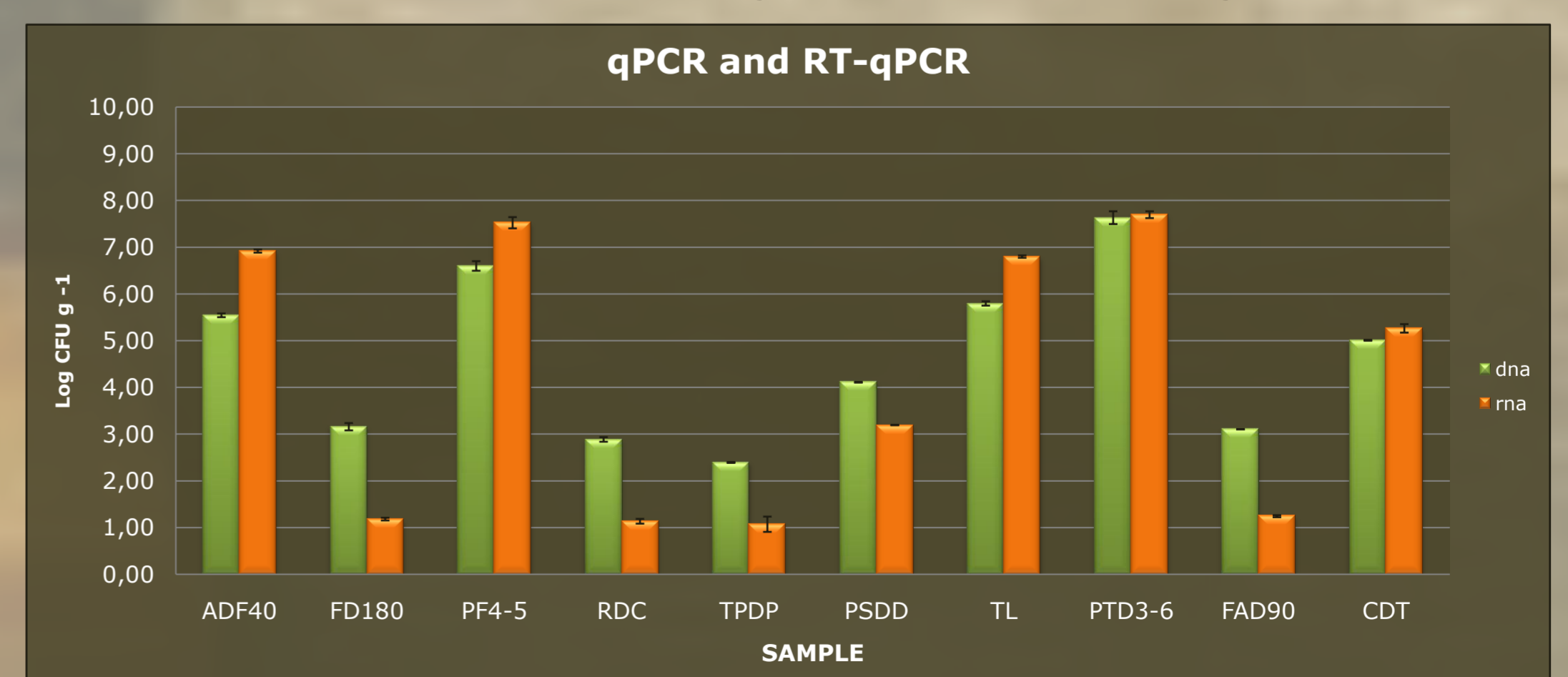
The results obtained by RT-qPCR and microbiological analysis were compared and were not always in agreement. In fact, the presence of *L. lactis* detected by RT-qPCR was not always confirmed by traditional plating and His-PCR. It could be hypothesized that M17 medium is not suitable to recovery *L. lactis* cells in stress conditions.

| COD.   | Plate counts Log CFU/g | His-PCR |
|--------|------------------------|---------|
| ADF40  | 6,94                   | 2       |
| FD180  | 6,38                   | 0       |
| PF4-5  | 7,79                   | 7       |
| RDC    | 6,40                   | 0       |
| TPDP   | 8,38                   | 0       |
| PSDD   | 7,76                   | 0       |
| TL     | 8,15                   | 1       |
| PTD3-6 | 6,51                   | 0       |
| FAD90  | 6,86                   | 0       |
| CDT    | 5,40                   | 1       |

Culture – independent approach

## 3. Application of the qPCR protocols in commercial cheeses

All the cheese samples (10 g) were homogenized with 40 mL of Ringer solution and an aliquot (1 mL) was subjected to both DNA and RNA extraction and qPCR and RT-qPCR. In ten of the cheeses analysed the load values were varying from 10<sup>1</sup> to 10<sup>7</sup> CFU/g. Specifically in four cheeses were higher than 10<sup>6</sup> CFU/g. In two of the cheeses (Bra PDO and Asiago d' Allevo PDO) no signal was detected.



## Conclusions

On the basis of our results, it can be concluded that the optimized qPCR protocols are suitable for the specific quantification of *L. lactis* species in cheese matrix, showing a good quantification limit of 10<sup>2</sup> CFU/g at both DNA and RNA level.

The culture-independent analysis highlighted the presence of the microorganism even at late ripening in contrast with traditional plating unables to detect microbial cells in VNC state.

A cheese-making pilot production will be assessed to follow the presence and activity of *L. lactis* from the inoculum in milk, as starter culture, until the end of the cheese ripening.

Culture – dependent approach