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Impact of extracellular nucleic acids from lactic acid bacteria on qPCR and RT-qPCR results in dairy matrices: Implications for defining molecular markers of cell integrity

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ABSTRACT

In this study, we evaluated whether quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR) results could be affected by extracellular (free) DNA or RNA, respectively, obtained either from isolated bacteria or from fermented dairy foods manufactured with these microorganisms. These experimental set-ups involved two starter lactic acid bacteria (SLAB), *Streptococcus thermophilus* and *Lactococcus lactis*, as models across their most common technological environments: yoghurt, milk and cheese. The presence of free DNA modified qPCR results in levels sufficient to bias SLAB determinations, indicating that DNA may be a poor measure of cellular integrity. Furthermore, we showed for the first time that an order of magnitude more of free RNA than of DNA had to be used to modify RT-qPCR results. Importantly, the interference produced by free DNA and RNA was maintained across all the dairy matrices evaluated, independently of the SLAB and of the source of nucleic acids used, in both negative and positive samples. This study suggests that, contrary to DNA molecules, mRNA from dead microorganisms or from other extracellular sources may not be a determinant factor in skewing molecular quantifications. Therefore, our results support the potential use of mRNA as a molecular signature of cell integrity in dairy matrices.

1. Introduction

Molecular (culture-independent) methods, including real-time quantitative PCR (qPCR) and reverse transcription-qPCR (RT-qPCR), have been extensively used to evaluate *Streptococcus thermophilus*, *Lactococcus lactis* and other lactic acid bacteria (LAB) in fermented dairy foods (Cocolin, Alessandria, Dolci, Gorra, & Rantsiou, 2013; Sohier, Pavan, Riou, Combrisson, & Postollec, 2014). *S. thermophilus* and *L. lactis* are both thermophilic LAB, traditionally used as dairy starters. These starter LAB (SLAB) possess important technological traits which make them the most important microorganisms in the dairy industry (Fernández, Alegría, Delgado, Martín, & Mayo, 2011; Mora & Arioli, 2014).

The study of microbial viability has been historically approached by culture-dependent methods and thus, if a microorganism could form colonies in culture media it was considered viable (Cangelosi & Meschke, 2014; Nocker & Camper, 2009). Nevertheless, unlike molecular methods, classical plating assays are incapable of detecting viable but nonculturable microorganisms (Ruggirello, Cocolin, & Dolci, 2016; Trevors, 2012). Among other molecular strategies, viability PCR and RT-qPCR have been proposed as alternatives to culture-dependent methods to discriminate between dead and viable cells (Davis, 2014; Trevors, 2012). However, due to the fact that these assays have shown limitations (Barbau-Piednoir et al., 2014; Birch, Dawson, Cornett, & Keer, 2001; Ju, Moyne, & Marco, 2016; Kobayashi, Oethinger, Tuohy, Hall, & Bauer, 2009; Scaturro et al., 2016; Sung, Hiett, & Stern, 2005; Taylor, Bentham, & Ross, 2014), the use of these methods to define accurate measures of cell viability is currently a subject of investigation (Cangelosi & Meschke, 2014; Davis, 2014; Elizaquível, Aznar, & Sánchez, 2014).

In the case of food and other complex matrices, it has been

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shown that optimal extraction of DNA or RNA from target bacteria requires the concentration of cells before nucleic acid purification (Stevens & Jaykus, 2004; Wilson, 1997). It is well known also that the long persistence of DNA after cell death makes it a poor measure of bacterial viability (Lauri & Mariani, 2009; Li, Zhao, Liu, Gao, & Zhang, 2013). However, it has been argued that working with pelleted cells before nucleic acid extraction would not allow the recovery of extracellular DNA or RNA from dead microorganisms (Achilleos & Berthier, 2013; Falentin et al., 2012). Until recently, no experimental evidence was available to test that hypothesis. However, we have recently shown that extracellular DNA from *S. thermophilus* can indeed affect the quantification of target cells by qPCR in negative milk samples, indicating that this method cannot be used to assess cell integrity in complex matrices (Pega et al., 2016).

In our previous work, the effect observed with free DNA from *S. thermophilus* on qPCR results was not observed on RT-qPCR results when equivalent amounts of free RNA from this SLAB were spiked into the same milk matrix (Pega et al., 2016). Therefore, the aim of this study was to further explore these findings to better evaluate the value of nucleic acids as biomarkers of bacterial integrity. For this purpose, we quantified the effect of extracellular DNA or RNA on qPCR or RT-qPCR results, respectively, obtained either from target bacteria in isolation or from these bacteria when active in fermented foods. These experimental set-ups used two SLAB (*S. thermophilus* and *L. lactis*) as models across their most common technological environments: yoghurt, milk and cheese for *S. thermophilus* and milk and cheese for *L. lactis*. Moreover, the influence of free nucleic acids on SLAB quantifications was determined both in negative and already positive dairy samples.

2. Materials and methods

2.1. Elaboration and sampling of experimental yoghurt

Stirred yoghurt was produced by following protocols optimized in our laboratory. Briefly, milk was fermented with *S. thermophilus* and *Lactobacillus delbrueckii* subsp. *bulgaricus*, using a package of DVS YC-X16 50U (CHR Hansen, Horsholm, Denmark). Then, 1 g of these starters was reconstituted into 100 mL of pasteurized skimmed milk (1.5 v/v) and thawed in a water bath at room temperature. Individually packed yoghurt samples were kept at 4 °C, sampled after 7 days of elaboration and stored at −80 °C until processing.

2.2. Elaboration and sampling of industrial cheese

Port Salut light cheeses were manufactured at an industrial plant as previously described (Pega et al., 2016). Another production batch was obtained with this protocol for *L. lactis* experiments, except that *L. lactis* subsp *lactis*, *subsp. cremoris* (Danisco Choozit MA14 LYO 50DCU, Copenhagen, Denmark) lyophilized starter culture was added together with *S. thermophilus* (STI-14 50U, CHR Hansen). Milk samples were obtained aseptically 30 min after the addition of the starter cultures. Vacuum-packed cheeses were ripened at 4 °C and sampled at 7 days of ripening. After collection, both milk and cheese samples were stored at −80 °C until processing.

2.3. Obtention of bacterial cells from milk, cheese and yoghurt samples

Bacterial cells were concentrated from milk and cheese samples as previously described (Pega et al., 2016). To obtain yoghurt pellets, 2-mL yoghurt samples were suspended in 20 mL 2% trisodium citrate - 4% polyethylene glycol 8000 (Sigma-Aldrich, Missouri, USA). The mixtures were centrifuged at 700×*g* for 10 min at 4 °C to discard pelleted material and supernatants were transferred to 50-mL tubes. Samples were centrifuged at 9700×*g* for 15 min at 4 °C. The supernatants were discarded and cell pellets were stored at −80 °C until nucleic acid extraction.

2.4. DNA extraction from starter cultures and from milk, cheese and yoghurt samples

DNA was purified as reported previously (Pega et al., 2016). Briefly, DNA was extracted by using DNAzol (Invitrogen, Massachusetts, USA), quantified with the Qubit 2.0 Fluorometer (dsDNA Assay BR Kit, Invitrogen) and stored at −80 °C.

2.5. RNA extraction from starter cultures and from milk, cheese and yoghurt samples

RNA was extracted as reported previously (Pega et al., 2016). Briefly, RNA was obtained by using TRIZol Reagent (Invitrogen) and quantified with the Qubit 2.0 Fluorometer (RNA Assay BR Kit, Invitrogen). Then, 1 μg of purified RNA samples was incubated for 15 min with 1 μL (1U/μL) of DNase I (Invitrogen). Residual DNA was checked by qPCR in controls prepared with non-retrotranscribed RNA.

2.6. Primer design and specificity determinations

S. thermophilus specific primers for DNA amplification in milk and cheese samples have been previously described (Pega et al., 2016). This same methodology was used for the design of *L. lactis* specific primers and for assessing *in silico* specificity. The nucleotide sequence of the primer pair was as follows: 5′- CAT CGT TGA TGA ATA CAT CCC AAC T - 3′ (f), and 5′- CGA CTG GAA GAA GGA GTG GTT T- 3′ (r). Cross amplification experiments to determine primer specificity for *L. lactis* were performed with 0.2–30 ng/μL of DNA extracted from *S. thermophilus* starter culture (STI-14 50U, CHR Hansen). Experiments to determine primer specificity for *S. thermophilus* (in yoghurt) and for *L. lactis* (in milk and cheese) were performed with 0.2–30 ng/μL of DNA from starter cultures containing *L. delbrueckii* subsp. *bulgaricus* (DVS YC-X16 50U, CHR Hansen) and *S. thermophilus* (STI-14 50U, CHR Hansen), respectively.

2.7. Real-time qPCR and RT-qPCR experiments

All the experimental procedures (addition of SLAB pellets, spiking of free nucleic acids) were performed in triplicate samples and then subjected to qPCR or RT-qPCR assays. These experiments were carried out in a StepOnePlus Real-Time PCR System (Applied Biosystems, CA, USA) as described previously (Pega et al., 2016). Reverse transcription was performed with the M-MLV Reverse Transcriptase Kit (Promega, WI, USA), using 5 μL of RNA and following the manufacturer's instructions.

2.8. Construction of standard curves for qPCR and RT-qPCR

The standard curves used for *S. thermophilus* DNA and cDNA quantification in milk and cheese samples have been previously described (Pega et al., 2016). The same protocol was followed to generate *S. thermophilus* standard curves in 2-mL yoghurt samples and to obtain *L. lactis* standard curves in milk and cheese samples. Briefly, pasteurized and autoclaved 20-mL milk and 2-g cheese samples were spiked with ten-fold amounts (0.04 μg - 40 mg) of *L. lactis* (Danisco Choozit MA14 LYO 50DCU) and subjected to DNA

and RNA extraction for qPCR or RT-qPCR assays. Standard curves were generated by interpolating Ct values (mean of triplicate samples) against the logarithm (log) number of genome or cDNA copies/mL or g of sample. The copy number was calculated as previously described (Pega et al., 2016).

3. Results

3.1. Assessment of primer specificity

To determine the primer specificity of *S. thermophilus* in yoghurt samples, DNA extracted from *L. delbrueckii* subsp. *bulgaricus* was tested and found to be below the detection threshold of the assay, even at the lowest dilution of template evaluated. The same results were obtained for cheese cross-reactivity assessments when *L. lactis* primers were tested against the DNA obtained from *S. thermophilus.* Moreover, the melting curves generated for *S. thermophilus* and *L. lactis* displayed only one peak in every sample, thus confirming the specificity of both primer pairs (data not shown).

3.2. Standard curves for S. thermophilus and L. lactis

Standard curves interpolated for *S. thermophilus* DNA and cDNA quantifications in yoghurt samples showed similar parameters than the standard curves previously used for milk and cheese samples (Pega et al., 2016). Correlation coefficients between Ct values and copy numbers presented R^2 values of 0.999 for both DNA and cDNA standard curves, whereas the detection spectrum was linear across a range of 7 log units. E% values were 92.35% for DNA and 93.23% for cDNA standard curves, respectively. The limit of detection (LOD) corresponded to 46 copies of DNA/well and 27 copies of cDNA/well.

Standard curves for *L. lactis* generated with negative cheese samples are shown in Fig. 1. Fifty-three copies of DNA/well and 50 copies of cDNA/well were accurately determined (LOD) in this matrix. Similar results were observed for standard curves performed with negative milk samples, where both DNA and cDNA standard curves also showed R^2 values of 0.999. In these samples, E % was 95.52% for DNA and 93.29% for cDNA curves, respectively, whereas LOD values were 76 copies of DNA/well and 89 copies of cDNA/well.

3.3. Experimental set-ups for negative dairy matrices spiked with target DNA or RNA

To evaluate whether qPCR and RT-qPCR outcomes could be affected by the presence of extracellular (free) nucleic acids from target microorganisms, negative milk (20 mL), yoghurt (2 mL) and cheese (2 g) samples were spiked with ten-fold dilutions of DNA (0.60 ng - 60 μg) or RNA (0.48 ng - 48 μg) obtained from *S. thermophilus*. This dilution range covered the amount of nucleic acids extracted from 22 mg of this SLAB (6.0 μg of DNA and 4.8 μg of RNA). In addition, the same SLAB was also spiked (22 mg of pellet) on another group of samples to serve as positive controls. Pellets harvested from all these samples were subjected to nucleic acid extraction for qPCR or RT-qPCR experiments (Fig. 2).

This experimental set-up was also applied to *L. lactis*, by spiking another group of negative milk and cheese samples with ten-fold dilutions of DNA (0.55 ng - 55 μg) or RNA (0.42 ng - 42 μg) (Fig. 3). The same as with *S. thermophilus* results, this dilution range covered the amount of nucleic acids extracted from 22 mg of *L. lactis* (5.5 μg of DNA and 4.2 μg of RNA).

As expected, the addition of SLAB to negative dairy samples was evidenced by qPCR in all of these matrices, with similar values for *S. thermophilus* (~2.7×10^5 copies/mL or g) and *L. lactis* (~2.4×10^5 copies/mL or g) (Figs. 2 and 3, panel A). In contrast, when negative dairy samples were spiked with 0.60–600 ng or 0.55–500 ng of DNA from *S. thermophilus* or *L. lactis*, respectively, no target nucleic acids were detected. Interestingly, when the same procedure was performed with one order of magnitude more of DNA, target nucleic acids from both SLAB were detected in all the dairy matrices evaluated. Similar values were obtained for *S. thermophilus* (~8.9×10^4 copies/mL or g) and *L. lactis* (~8.2×10^4 copies/mL or g) (Figs. 2 and 3, panel A). Accordingly, the amount of target nucleic acids detected from both SLAB also increased in all the dairy matrices tested after negative dairy samples were spiked with an additional order of magnitude of SLAB DNA (60 μg from *S. thermophilus* or 55 μg from *L. lactis*). As expected, these values were above the detection threshold of the assay for both SLAB (Figs. 2 and 3, panel A).

The addition of SLAB to negative dairy samples produced high amounts of cDNA copies by RT-qPCR in all the matrices, with similar values for *S. thermophilus* (~2.0×10^5 copies/mL or g) and *L. lactis* (~1.9×10^5 copies/mL or g) (Figs. 2 and 3, panel B). In contrast, when negative dairy samples were spiked with 0.48–4.8 μg or 0.42–4.2 μg of RNA from *S. thermophilus* or *L. lactis*, respectively, no target nucleic acids were detected in any of the dairy matrices evaluated. However, when the same procedure was performed with one order of magnitude more of RNA, target nucleic acids were detected for both SLAB in all the matrices tested. The same as with qPCR results, similar values were observed for *S. thermophilus* (~7.3×10^4 copies/mL or g) and *L. lactis* (~7.5×10^4 copies/mL or g) (Figs. 2 and 3, panel B).

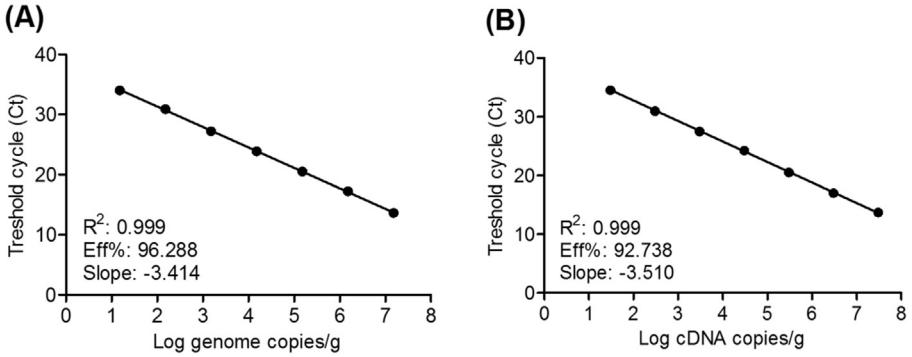

Fig. 1. Standard curves generated for *Lactococcus lactis* by qPCR (panel A) and RT-qPCR (panel B). Each point represents the mean value of triplicate DNA or RNA extractions ± standard deviations (SD). Standard curves were constructed by plotting threshold cycle (Ct) values against the logarithm (log) number of genome or cDNA copies/g of negative cheese samples.

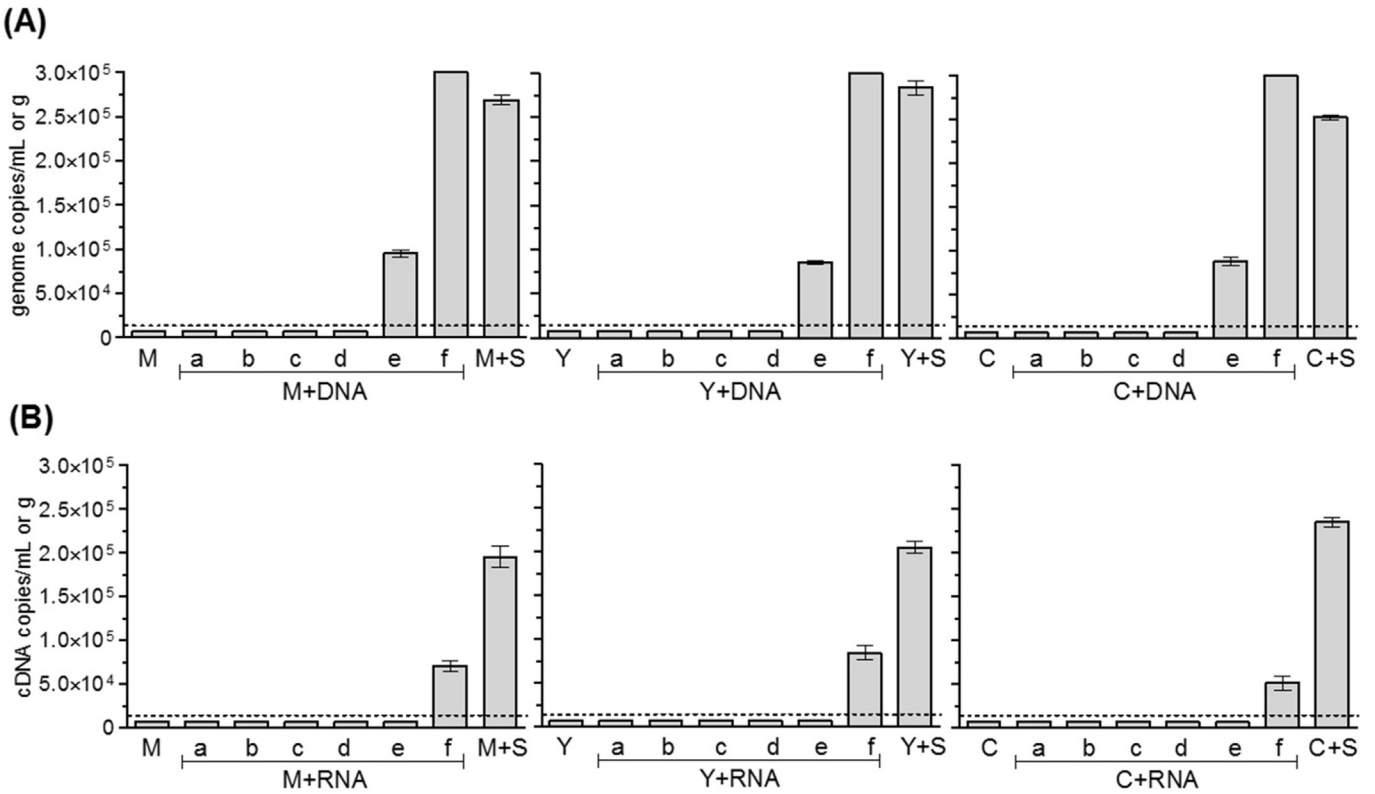

Fig. 2. Quantification of *Streptococcus thermophilus* by qPCR (panel A) and RT-qPCR (panel B) in negative dairy matrices spiked with nucleic acids obtained from the lyophilized starter. M: negative milk. **Y:** negative yoghurt. **C:** negative cheese. **S:** *S. thermophilus* starter culture. Letters **a** to **f** correspond to ten-fold dilutions of DNA (0.60 ng - 60 μg) or RNA (0.48 ng - 48 μg). Results are expressed as the number of genome or cDNA copies/mL or g of sample. Each bar represents the mean value of triplicate samples ± SD. The detection limit of the assay is represented by a dotted line.

3.4. Experimental set-up for negative dairy matrices spiked with DNA or RNA obtained from fermented dairy foods

In an effort to determine whether extracellular DNA or RNA from SLAB could modify the quantifications of these bacteria in real food fermentations, experimental stirred yoghurt and industrial low-fat cheese were both manufactured with *S. thermophilus*. This cheesemaking process was also repeated with *L. lactis*. Milk samples were obtained 30 min after the addition of the SLAB during both cheesemaking batches, while yoghurt and ripened cheese samples were obtained after 7 days of elaboration (positive samples used both as sources of nucleic acids and as controls).

Negative milk, yoghurt and cheese samples were spiked with DNA or RNA obtained from pellets of positive samples, in the same dilutions used for the previous experiments (section 3.3). Pellets from these and from positive samples (controls) were subjected to nucleic acid purification for qPCR or RT-qPCR experiments (Figs. 4 and 5).

As expected, all dairy foods elaborated with SLAB (positive samples) showed presence of target nucleic acids (Figs. 4 and 5, panel A). In these foods, ~2.2 × 10^5 genome copies of *S. thermophilus*/mL (milk, yoghurt) or g (cheese) and ~1.8 × 10^5 genome copies of *L. lactis*/mL (milk) or g (cheese) were detected. In contrast, when negative dairy samples were spiked with 0.60–600 ng or 0.55–500 ng of DNA from *S. thermophilus* or *L. lactis*, respectively, no target nucleic acids were detected (data not shown). However, when the same procedure was performed with one order of magnitude more of DNA, target nucleic acids from both SLAB were detected in all the dairy matrices evaluated. Similar values were obtained for *S. thermophilus* (~9.1 × 10^4 copies/mL or g) and *L. lactis* (~7.3 × 10^4 copies/mL or g) (Figs. 4 and 5, panel A). Accordingly, when negative dairy samples were spiked with an additional order of magnitude of DNA from positive samples (60 μg from *S. thermophilus* or 55 μg from *L. lactis*), the amount of target nucleic acids detected from both SLAB also increased in all the dairy matrices evaluated. As expected, these values were above the detection threshold of the assay for both SLAB (data not shown).

The same as with qPCR experiments, all dairy foods elaborated with *S. thermophilus* and *L. lactis* showed presence of target nucleic acids by RT-qPCR (Figs. 4 and 5, panel B). In these foods, ~1.6 × 10^5 cDNA copies of *S. thermophilus*/mL (milk, yoghurt) or g (cheese) and ~1.4 × 10^5 cDNA copies of *L. lactis*/mL (milk) or g (cheese) were detected. When negative dairy samples were spiked with 0.48–4.8 μg or 0.42–4.2 μg of RNA from *S. thermophilus* or *L. lactis*, respectively, no target nucleic acids were detected in any of the dairy matrices evaluated (data not shown). However, when the same procedure was performed with one order of magnitude more of RNA, target nucleic acids were detected, although in low amounts for both SLAB. Almost the same values were observed for *S. thermophilus* and *L. lactis* cDNA levels (~4.9 × 10^4 copies/mL or g) (Figs. 4 and 5, panel B).

3.5. Experimental set-up for positive dairy matrices spiked with DNA or RNA obtained from fermented dairy foods

To determine the extent to which qPCR and RT-qPCR results could be biased by the presence of extracellular nucleic acids in already positive samples (elaborated with SLAB), the latter were spiked with DNA or RNA obtained from replicates of themselves. Nucleic acids were spiked by using the minimum amounts that could be accurately quantified when tested previously in negative samples (section 3.4). In addition, another group of these samples

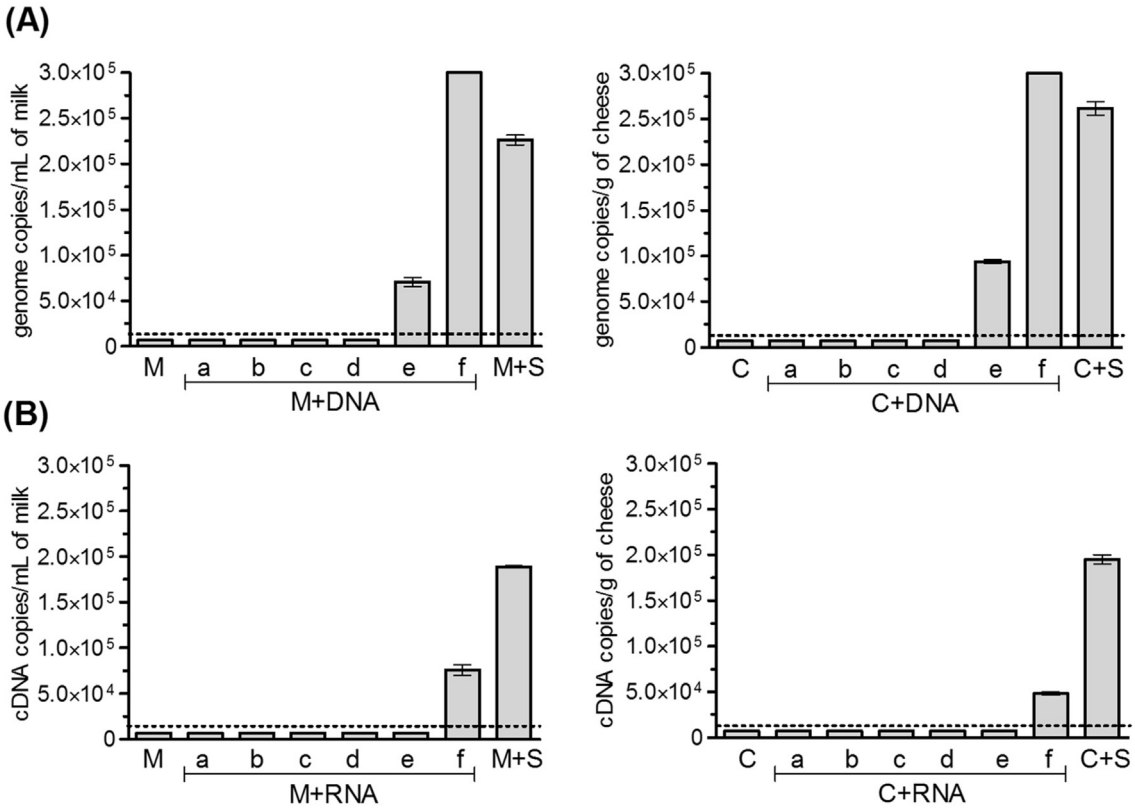

Fig. 3. Quantification of *Lactococcus lactis* by qPCR (panel A) and RT-qPCR (panel B) in negative dairy matrices spiked with nucleic acids obtained from the lyophilized starter. M: negative milk. **C:** negative cheese. **S:** *L. lactis* starter culture. Letters **a** to **f** correspond to ten-fold dilutions of DNA (0.55 ng - 55 μg) or RNA (0.42 ng - 42 μg). Results are expressed as in Fig. 2.

were spiked with pellets from replicates of themselves to serve as positive controls for these experiments. Pellets harvested from all these samples were subjected to nucleic acid purification for qPCR or RT-qPCR experiments (Figs. 4 and 5).

The amount of target nucleic acids detected for both SLAB was higher in all positive dairy samples when these had been spiked with DNA (~25% increase in genome copies) (Figs. 4 and 5, panel A). As expected, positive samples showed very high levels of nucleic acids after the addition of pellets from replicates of themselves (genome copies were outside the linear quantification range).

Similar to qPCR results, the amount of target cDNA detected was higher in all positive dairy samples when these had been spiked with RNA (Figs. 4 and 5, panel B). Also as expected, positive samples showed high levels of cDNA after the addition of pellets from replicates of themselves (~2.7 × 10^5 copies/mL or g).

4. Discussion

The study of DNA and mRNA as indirect estimates of microbial integrity or viability was performed in the past mainly through the use of viable and killed bacteria in isolation (Cangelosi & Meschke, 2014; Davis, 2014; Elizaquível et al., 2014; Kort, Keijser, Caspers, Schuren, & Montijn, 2008; Nocker & Camper, 2009; Trevors, 2012). Therefore, there is limited experimental evidence on how extracellular nucleic acids from sources other than target cells can affect DNA and mRNA quantifications in complex matrices (Pega et al., 2016). Consequently, although molecular methods have been extensively used in the field of microbiology (Cocolin et al., 2013; Sohier et al., 2014), information on the usefulness of qPCR or RT-qPCR for assessing bacterial integrity in complex matrices remains scarce. Here, we approached the evaluation of target nucleic acids as molecular biomarkers of bacterial integrity by quantifying the effect of extracellular DNA or RNA on qPCR or RT-qPCR results, respectively, obtained either from bacteria in isolation or from these bacteria when active in fermented foods.

In the present work, 6.0 μg of DNA extracted from *S. thermophilus* or 5.5 μg from *L. lactis*, respectively, were required to detect target nucleic acids by qPCR when spiked in negative dairy matrices. For both SLAB DNA, the number of genome copies was ~60% lower than the one recorded for the corresponding SLAB pellets containing equivalent amounts of nucleic acids, supporting our previous findings (Pega et al., 2016). Accordingly, the number of genome copies recorded in that study was ~56% lower for SLAB DNA than for SLAB pellets. Although both studies highlighted that the detection threshold of prokaryotic DNA was much lower for extracellular than for intracellular DNA, they demonstrated that it was possible to detect free DNA by qPCR in pelleted microorganisms.

Importantly, these results were not dependent on the SLAB, on the source of nucleic acids or on the dairy matrix evaluated in this study. Moreover, the effect observed with free DNA from real dairy foods on negative dairy samples was also observed in already positive samples (~25% increase in DNA detection levels), suggesting that extracellular DNA can bias qPCR results both in the presence and in the absence of target bacteria (intracellular DNA). Therefore, the ability of free DNA to modify qPCR results in the levels recorded here and in our previous experimental model (Pega et al., 2016) indicates that the DNA detected by qPCR assays may be a poor measure of cellular integrity, even if working with pelleted microorganisms from complex matrices before nucleic acid extraction.

Here, when negative dairy matrices were spiked with

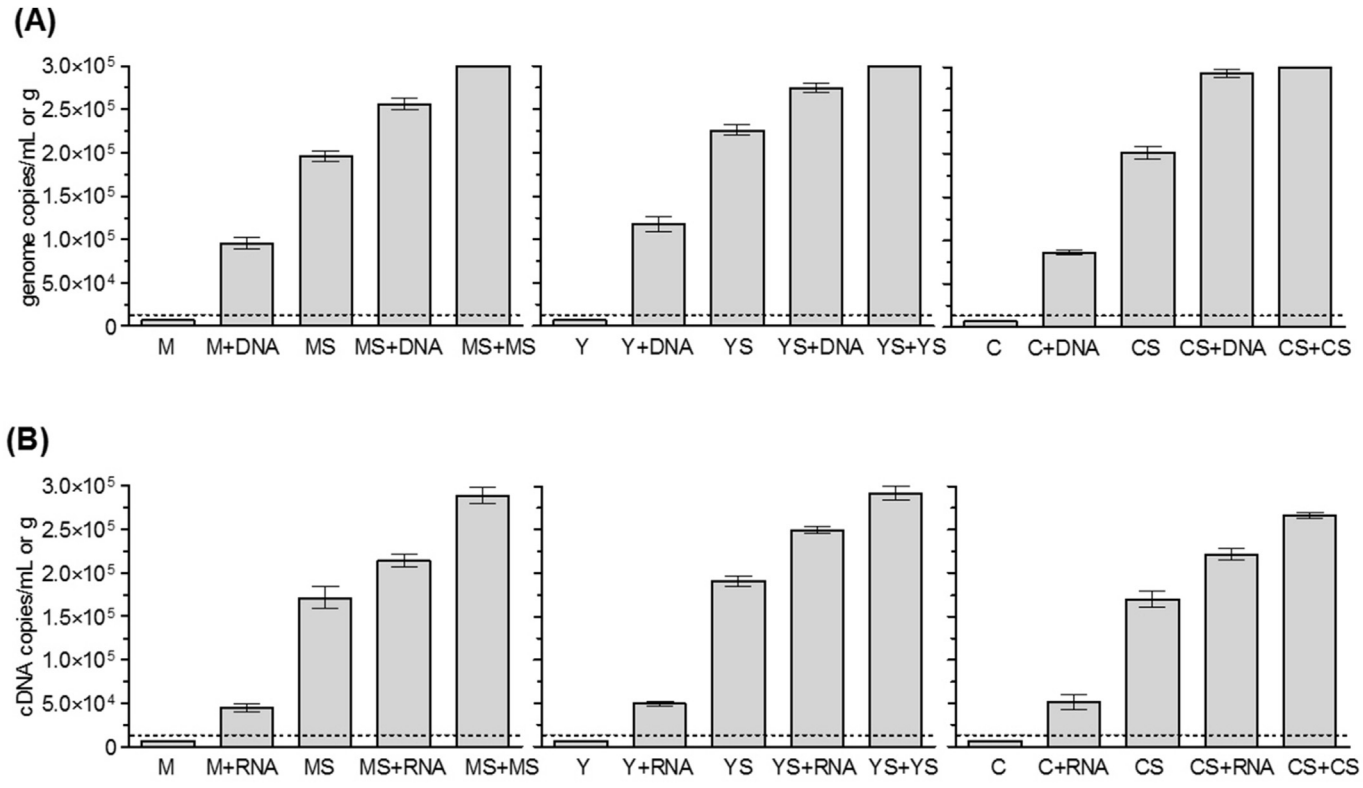

Fig. 4. Quantification of *Streptococcus thermophilus* by qPCR (panel A) and RT-qPCR (panel B) in negative and positive dairy matrices spiked with nucleic acids obtained from fermented foods. M: negative milk. **Y:** negative yoghurt. **C:** negative cheese. **MS**, **YS**, **CS:** milk, yoghurt and cheese elaborated with the starter *S. thermophilus*, respectively (positive samples). Results are expressed as in Fig. 2.

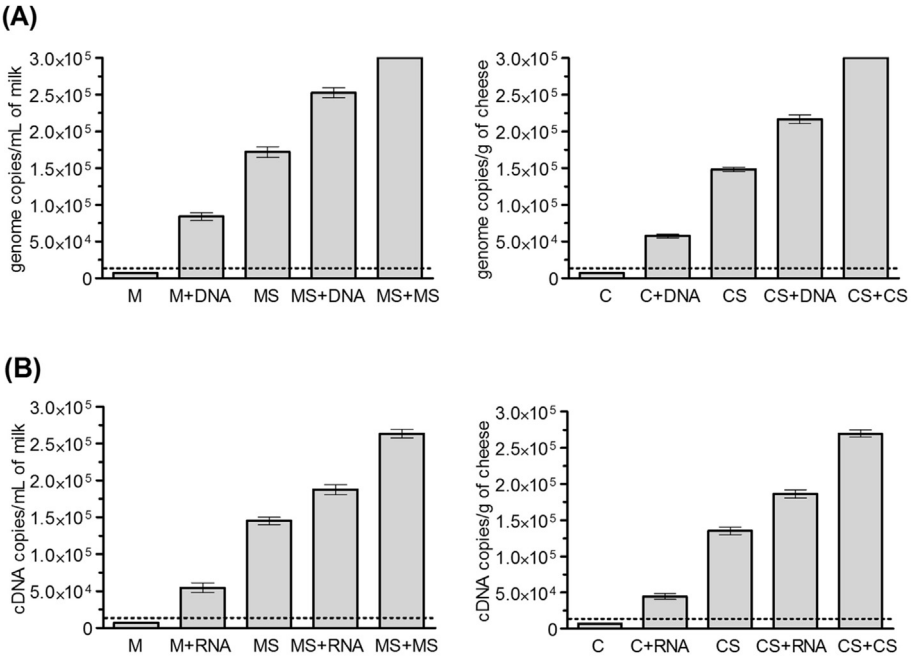

Fig. 5. Quantification of *Lactococcus lactis* by qPCR (panel A) and RT-qPCR (panel B) in negative and positive dairy matrices spiked with nucleic acids obtained from fermented foods. M: negative milk. **C:** negative cheese. **MS**, **CS:** milk and cheese elaborated with the starter *L. lactis*, respectively (positive samples). Results are expressed as in Fig. 2.

0.48–4.8 μg or 0.42–4.2 μg of RNA from *S. thermophilus* or *L. lactis*, respectively, no target nucleic acids were detected. This is in agreement with our previous findings (Pega et al., 2016), where *S. thermophilus* RNA could not be detected by RT-qPCR, in the same orders of magnitude, when spiked in negative milk samples. Interestingly, 48 μg of RNA from *S. thermophilus* or 42 μg from *L. lactis*, respectively, had to be spiked in negative dairy matrices to detect target nucleic acids by RT-qPCR. This amount corresponded

to an order of magnitude more than the amount of RNA contained in SLAB pellets (positive controls) and than the relative amount of free DNA required to affect the detection threshold. Importantly, the same as with free DNA patterns, this effect was not dependent on the SLAB, on the source of nucleic acids or on the dairy matrix evaluated in this study.

Prokaryotic mRNA can sometimes persist after cell death for variable periods of time depending on numerous factors and on the experimental approaches used (Birch et al., 2001; Davis, 2014; Ju et al., 2016; Kort et al., 2008; Lauri & Mariani, 2009; Sheridan, Szabo, & Mackey, 1999; Sung, Stern, & Hiett, 2004; Sung et al., 2005). Consequently, since mRNA is not always correlated to the presence of viable bacteria, its detection in this study does not unequivocally prove cell viability. However, unlike the experiments performed with DNA, the insignificant interference produced by free RNA on RT-qPCR results suggests that mRNA from sources other than target cells may not affect molecular quantifications.

The differences observed between qPCR and RT-qPCR results outlined above and their consistence with previous knowledge regarding nucleic acid stability (Hellyer et al., 1999; Klein & Juneja, 1997; Li et al., 2013; Sheridan et al., 1999; Zhao, Yao, & Hsing, 2006) suggest that extracellular mRNA experienced a higher decay rate than the mRNA contained in bacterium pellets or than free DNA molecules. Consequently, although it cannot be known for sure if the mRNA was obtained from viable or from death microorganisms, our results demonstrate that its detection by RT-qPCR did not correspond to free nucleic acids when realistic amounts of RNA were used. These findings indicate that it is possible to exploit these differences in environmental stability between free mRNA and the mRNA contained in bacterium cells, in order to estimate cell integrity in dairy foods and possibly in other complex matrices.

In summary, our results showed that free DNA modified qPCR results in levels sufficient to bias determinations of the SLAB *S. thermophilus* and *L. lactis* in all the dairy matrices tested, independently of their source and in both negative and positive samples. This extended previous findings showing that the DNA detected by qPCR may be a poor measure of cellular integrity, even in complex matrices which involve pelleted microorganisms before nucleic acid purification. Furthermore, this study highlighted for the first time marked differences in detection levels between extracellular and intracellular mRNA. Therefore, although further studies are needed to better define correlations between specific mRNA transcripts and metabolic activity or viability, our results support the possible use of RT-qPCR as an accurate molecular measure of bacterial integrity in dairy matrices.

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References

Achilleos, C., & Berthier, F. (2013). Quantitative PCR for the specific quantification of *Lactococcus lactis* and *Lactobacillus paracasei* and its interest for *Lactococcus lactis* in cheese samples. *Food Microbiology, 36*(2), 286–295.

Barbau-Piednoir, E., Mahillon, J., Pillyser, J., Coucke, W., Roosens, N. H., & Botteldoorn, N. (2014). Evaluation of viability-qPCR detection system on viable and dead *Salmonella* serovar enteritidis. *Journal of Microbiological Methods, 103*, 131–137.

Birch, L., Dawson, C. E., Cornett, J. H., & Keer, J. T. (2001). A comparison of nucleic acid amplification techniques for the assessment of bacterial viability. *Letters in Applied Microbiology, 33*(4), 296–301.

Cangelosi, G. A., & Meschke, J. S. (2014). Dead or alive: Molecular assessment of microbial viability. *Applied and Environmental Microbiology, 80*(19), 5884–5891.

Cocolin, L., Alessandria, V., Dolci, P., Gorra, R., & Rantsiou, K. (2013). Culture independent methods to assess the diversity and dynamics of microbiota during food fermentation. *International Journal of Food Microbiology, 167*(1), 29–43.

Davis, C. (2014). Enumeration of probiotic strains: Review of culture-dependent and alternative techniques to quantify viable bacteria. *Journal of Microbiological Methods, 103*, 9–17 (Review).

Elizaquível, P., Aznar, R., & Sánchez, G. (2014). Recent developments in the use of viability dyes and quantitative PCR in the food microbiology field. *Journal of Applied Microbiology, 116*(1), 1–13 (Review).

Falentin, H., Henaff, N., Le Bivic, P., Deutsch, S. M., Parayre, S., Richoux, R., et al. (2012). Reverse transcription quantitative PCR revealed persistency of thermophilic lactic acid bacteria metabolic activity until the end of the ripening of Emmental cheese. *Food Microbiology, 29*, 132–140.

Fernández, E., Alegría, A., Delgado, S., Martín, M. C., & Mayo, B. (2011). Comparative phenotypic and molecular genetic profiling of wild *Lactococcus lactis* subsp. *lactis* strains of the *L. lactis* subsp. *lactis* and *L. lactis* subsp. *cremoris* genotypes, isolated from starter-free cheeses made of raw milk. *Applied and Environmental Microbiology, 77*(15), 5324–5335.

Hellyer, T. J., DesJardin, L. E., Teixeira, L., Perkins, M. D., Cave, M. D., & Eisenach, K. D. (1999). Detection of viable *Mycobacterium tuberculosis* by reverse transcriptase-strand displacement amplification of mRNA. *Journal of Clinical Microbiology, 37*, 518–523.

Ju, W., Moyne, A. L., & Marco, M. L. (2016). RNA-Based detection does not accurately enumerate living *Escherichia coli O157:H7* cells on plants. *Frontiers in Microbiology, 7*, 223.

Klein, P. G., & Juneja, V. K. (1997). Sensitive detection of viable *Listeria monocytogenes* by reverse transcription-PCR. *Applied and Environmental Microbiology, 63*, 4441–4448.

Kobayashi, H., Oethinger, M., Tuohy, M. J., Hall, G. S., & Bauer, T. W. (2009). Unsuitable distinction between viable and dead *Staphylococcus aureus* and *Staphylococcus epidermidis* by ethidium bromide monoazide. *Letters in Applied Microbiology, 48*(5), 633–638.

Kort, R., Keijser, B. J., Caspers, M. P., Schuren, F. H., & Montijn, R. (2008). Transcriptional activity around bacterial cell death reveals molecular biomarkers for cell viability. *BMC Genomics, 9*, 590.

Lauri, A., & Mariani, P. O. (2009). Potentials and limitations of molecular diagnostic methods in food safety. *Genes & Nutrition, 4*(1), 1–12.

Li, M., Zhao, G., Liu, J., Gao, X., & Zhang, Q. (2013). Effect of different heat treatments on the degradation of *Salmonella* nucleic acid. *Journal of Food Safety, 33*, 536–544.

Mora, D., & Arioli, S. (2014). Microbial urease in health and disease. *PloS One Pathogens, 10*(12), e1004472.

Nocker, A., & Camper, A. K. (2009). Novel approaches toward preferential detection of viable cells using nucleic acid amplification techniques. *FEMS Microbiology Letters, 291*(2), 137–142.

Pega, J., Rizzo, S., Pérez, C. D., Rossetti, L., Díaz, G., Ruzal, S. M., et al. (2016). Effect of the addition of phytosterols and tocopherols on *Streptococcus thermophilus* robustness during industrial manufacture and ripening of a functional cheese as evaluated by qPCR and RT-qPCR. *International Journal of Food Microbiology, 232*, 117–125.

Ruggirello, M., Cocolin, L., & Dolci, P. (2016). Fate of *Lactococcus lactis* starter cultures during late ripening in cheese models. *Food Microbiology, 59*, 112–118.

Scaturro, M., Fontana, S., Dell'eva, I., Helfer, F., Marchio, M., Stefanetti, M. V., et al. (2016). A multicenter study of viable PCR using propidium monoazide to detect *Legionella* in water samples. *Diagnostic Microbiology and Infectious Disease, 85*(3), 283–288.

Sheridan, G. E., Szabo, E. A., & Mackey, B. M. (1999). Effect of post-treatment holding conditions on detection of tufA mRNA in ethanol-treated *Escherichia coli*: Implications for RT-PCR-based indirect viability tests. *Letters in Applied Microbiology, 29*(6), 375–379.

Sohier, D., Pavan, S., Riou, A., Combrisson, J., & Postollec, F. (2014). Evolution of microbial analytical methods for dairy industry needs. *Frontiers in Microbiology, 5*, 16.

Stevens, K. A., & Jaykus, L. A. (2004). Direct detection of bacterial pathogens in representative dairy products using a combined bacterial concentration-PCR approach. *Journal of Applied Microbiology, 97*, 1115–1122.

Sung, K., Hiett, K. L., & Stern, N. J. (2005). Heat-treated *Campylobacter spp.* and mRNA stability as determined by reverse transcriptase-polymerase chain reaction. *Foodborne Pathogens and Disease, 2*(2), 130–137.

Sung, K. D., Stern, N. J., & Hiett, K. L. (2004). Relationship of messenger RNA reverse transcriptase-polymerase chain reaction signal to *Campylobacter spp.* viability. *Avian Diseases, 48*(2), 254–262.

Taylor, M. J., Bentham, R. H., & Ross, K. E. (2014). Limitations of using propidium monoazide with qPCR to discriminate between live and dead *Legionella* in biofilm samples, 2014 Sep. 4 *Microbiology Insights, 7*, 15–24.

Trevors, J. T. (2012). Can dead bacterial cells be defined and are genes expressed after cell death? *Journal of Microbiological Methods, 90*(1), 25–28.

Wilson, I. G. (1997). Inhibition and facilitation of nucleic acid amplification. *Applied and Environmental Microbiology, 63*(10), 3741–3751 (Review).

Zhao, W., Yao, S., & Hsing, I. M. (2006). A microsystem compatible strategy for viable *Escherichia coli* detection. *Biosensors & Bioelectronics, 21*, 1163–1170.