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Zoom on starter lactic acid bacteria development into oxytetracycline spiked ovine milk during the early acidification phase

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ABSTRACT

Residues of antibiotics used in food-producing animals can be excreted in milk, affecting the technological performance of starter lactic acid bacteria (SLAB). SLAB development and acidification performance was studied in thermised ovine milk spiked with oxytetracycline (OTC) at maximum residue limit (MRL, 100 μg kg^{-1}), during the early acidification phase. Late milk acidification and lower lactic acid concentration revealed an antibiotic effect mainly after 6 and 7 h from inoculation, with a 6 h delay to reach pH 5.6; the delay in SLAB development was observed by BactoScan™, while viable counts were not affected. Real-time PCR evidenced OTC effect for *Streptococcus thermophilus* and *Lactobacillus helveticus*, not for *Lactobacillus delbrueckii* subsp. *lactis*. A statistically significant impact of the antibiotic was observed, even at the maximum residue limit, with possible repercussions in technological processing and in human health, due to an increase of its concentration in cheese.

1. Introduction

The continuous and indiscriminate administration of antibiotics to animals, particularly those entering the food chain, can have an impact on human health and animal origin food production (Chang, Wang, Regev-Yochay, Lipsitch, & Hanage, 2015; Heuer, Schmitt, & Smalla, 2011; Khosrokhavar et al., 2008; Smith, Dushoff, & Morris, 2005; Willis, 2000). In the last several years, the presence of antibiotics throughout the food chain has constituted a threat regarding the possible development of transferable antibiotic resistance not only in pathogens, but even in commensal bacteria, including lactic acid bacteria (LAB) (Mathur & Singh, 2005; Roca et al., 2015). Moreover, from a technological point of view, the presence of antibiotics in milk could cause a delayed fermentative process during yoghurt and cheese manufacturing, and altered sensory proprieties of the cheese (Beltrán, Morari-Pirlog, Quintanilla, Escriche, & Molina, 2018; Berruga, Molina, Althaus, & Molina, 2016).

Among the veterinary antibiotics, the tetracyclines class is the most sold, representing 33% of the antimicrobial agents administered in Europe livestock, and in Italy accounted for 27.1% of total antibiotic sales (EMA, 2016). Oxytetracycline (OTC), belonging to tetracycline class, exerts a bacteriostatic action through the inhibition of protein synthesis by reversibly binding of the 30S ribosome subunit (Chopra & Roberts, 2001). OTC is commonly administered to food-producing animals, and it can be partially excreted through milk (Aalipour, Mirlohi, Jalali, & Azadbakht, 2015; Naik et al., 2017; Tona & Olusola, 2014).

The Annex to Commission Regulation (EU) No. 37/2010 (EC, 2010) fixed the maximum residue limit (MRL) for tetracyclines at 100 μg kg^{-1} for milk, while no limits are established for milk-derived products.

Several studies dealing with the effect of OTC on dairy products manufacturing are present in literature (Berruga, Beltrán, Novés, Molina, & Molina, 2012; Berruga et al., 2016; Cabizza et al., 2017, 2018), but LAB development was not deeply investigated during the first hours after starter inoculation.

This study focussed on the behaviour of commercial SLAB (namely, *Streptococcus thermophilus, Lactobacillus helveticus*, and *Lactobacillus delbrueckii* subsp. *lactis*) during the early acidification

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by monitoring their development and acidification performance in OTC-spiked ovine milk at the MRL level.

2. Materials and methods

2.1. Experimental plan

Ovine milk was collected from an experimental flock of Sarda breed sheep at AGRIS Sardegna Research Agency (Sassari, Italy). Sheep were in good health and did not undergo antibiotic treatment. The milk was divided into three aliquots of 1.1 L each. The first lot was the control (not spiked, CTR), the second was spiked with OTC at MRL level (100 μg kg^{-1}), and the third was used only for thermisation temperature monitoring. Subsequently, the three aliquots of raw milk were heated to 63 °C in a water bath, then immediately cooled to 38 °C, in ice water. The starter culture (CHOOZIT® Su Casu LYO, Danisco, Denmark), composed of *S. thermophilus*, *Lb. delbrueckii* subsp. *lactis*, and *Lb. helveticus*, was inoculated at about 6 log colony forming units (cfu) g^{-1}. Then, both CTR and OTC spiked aliquots were divided into five sub-aliquots of 200 mL each, kept at constant temperature of 35 ± 1 °C in a water bath, whereas the remaining 100 mL were immediately used for the analyses performed after inoculum of the starter culture. Four CTR and four OTC spiked sub-aliquots of thermised milk, before and immediately after the starter addition (time 0), and at 4, 5, 6, and 7 h from inoculation, were sampled. The remaining two sub-aliquots of 200 mL (one CTR and one OTC spiked) were left in the water bath for continuous pH and temperature monitoring until 22 h post-inoculation. The experiment was replicated three times in a short period of eight days (on April 2018) to minimise possible effects due to milk composition.

2.2. Acidification curves

Acidification of CTR and OTC spiked milk samples was performed in sterile glass bottles, and temperature and pH were continuously monitored and recorded by an eight channel Liquiline CM448 transmitter (Endress + Hauser, Gerlingen, Germany) coupled with Field Data Manager Software (v. 1.03, Endress + Hauser).

2.3. Lactic acid determination

The lactic acid determination was performed by HPLC-RID analysis after clarification of milk samples, using an Agilent 1100 series HPLC system. Briefly, 1 g whole milk was added to 0.5 mL Carrez I reagent (36 g L^{-1} $FeK_3(CN)_6 \cdot 3H_2O$) (Carlo Erba, Milan, Italy), 0.5 mL Carrez II reagent (72 g L^{-1} $ZnSO_4 \cdot 7H_2O$) (Carlo Erba) and diluted with water to a final volume of 10 mL. The solution was vortexed for 1 min, then centrifuged at 4000 ×*g* for 10 min using a Centrifuge Neya 16R (Remi Elektrotechnik LTD, Vasai, India).

The supernatant was then filtered with 0.2 μm cellulose syringe filter and injected into HPLC. Chromatographic separation was achieved with a Rezex ROA − Organic Acid H^+ column 300 × 7.8 mm (Phenomenex, Torrance, CA, USA), under isocratic conditions, using 0.005 N H_2SO_4 in water as mobile phase. The flow was set to 0.450 mL min^{-1} and the column oven temperature was set at 28 °C. Identification of lactic acid was obtained by comparison of the retention time of the relevant peak with the standard's peak. Lactic acid quantification was performed by external calibration in the range 0.01–1 g L^{-1} using a lactic acid analytical standard (Supelco, St. Louis, MO, USA). The instrumental limit of detection (LOD), calculated from the calibration function according to the Hubaux-Vos method (Hubaux & Vos, 1970), was 0.0075 ± 0.0005 g L^{-1}. The typical recovery, calculated on spiked ovine milk in the range 0.02–0.4 g L^{-1}, was 100 ± 2%.

2.4. Viable bacterial counts

CTR and OTC milk samples were collected at the time points listed above. One mL of each serial ten-fold dilution performed in sterile saline solution (0.89%, w/v, NaCl), was seeded in a duplicate set of plates. Viable counts of thermophilic starter cocci and lactobacilli were performed in M17 agar (Microbiol, Uta (CA), Italy), incubated in aerobiosis for 48 h at 37 °C, and in MRS agar pH 5.4 (Microbiol), incubated in anaerobiosis (Oxoid™ AnaeroGen™, ThermoFisher Scientific, Rodano, Italy) for 48 h at 44 °C. Microbial counts were expressed as average ± SD log cfu mL^{-1}.

2.5. Individual bacterial counts (IBC)

The total number of bacterial cells was determined at the ARA Sardegna milk and food laboratory (Oristano, Italy), according to an internal validated method recognised by the Italian Accreditation Body (ACCREDIA), and following the ISO/IEC 17025 standards (ISO, 2017). Briefly, cold milk samples were manually stirred and positioned into a BactoScan™ FC 150 (FOSS Electric, Hillerød, Denmark) that automatically performed cell lysis and break down of interfering components, DNA fluorochrome staining, and flow cytometry with fluorescence detection (excitation 510 nm, emission 595 nm).

2.6. DNA extraction

Total community DNA from CTR and OTC milk was extracted at 0, 4, 5, 6, and 7 h after incubation. One millilitre of each milk sample was placed in 2 mL Eppendorf tubes supplemented with 600 μL 400 mM NaOH and 300 μL trisodium citrate dehydrate solution (40%, w/v), then vigorously vortexed for 30 s, and rested for 15 min. The tubes were centrifuged at 13,000 ×*g* at 4 °C for 15 min using a Centrifuge Eppendorf 5430 R (Eppendorf, Milan, Italy). Then, the supernatant and the upper fat layer were removed and the pellet was frozen at −80 °C until DNA extraction. Total community DNA was extracted by the DNeasy PowerFood Microbial Kit (Qiagen, Milan, Italy), and quantified by NanoDrop spectrophotometer (ThermoFisher Scientific, Milan, Italy), then stored at −80 °C.

2.7. Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was performed on the total community DNA extracted from milk at the selected time points (0, 4, 5, 6 and 7 h). The quantification of *S. thermophilus*, *Lb. helveticus* and *Lb. delbrueckii* subsp. *lactis* was based on the determination of the abundance of the genes *lacZ*, *prtH* and *dppE*, respectively. The primers used were designed by Cremonesi et al. (2011). For all the genes targeted was used the same qPCR mastermix composition of 20 μL, except the primers: 1 × Pol B buffer (EURx, Gdańsk, Poland), 3 mM $MgCl_2$, 0.2 mM DNTPs, 1 × EvaGreen dye (Biotium Inc., Hayward, CA, USA), 0.5 U Taq (EURx) and 0.4 μM of each primer. The qPCRs were performed by iQ5 Real-Time PCR Detection System (Bio-Rad, Hercules, CA, USA), and the protocol used was the same for all the three genes tested: 5 min at 95 °C, followed by 45 cycles of 15 s at 95 °C and 58 °C for 30 s. A final elongation step for 7 min at 72 °C was also performed. A qPCR standard curve for each target gene was generated by amplifying serial ten-fold dilutions of each PCR product freshly prepared, isolated from agarose gel (1.5%, w/v), purified by Wizard® SV Gel and PCR Clean-up System (Promega, Milan, Italy), then verified for purity by agarose gel electrophoresis

(1.5%, w/v), and quantified by NanoDrop spectrophotometer (ThermoFisher Scientific).

2.8. Statistical analysis

Acidification curves were processed with Prism (v. 7, GraphPad, La Jolla, CA, USA), and linear regression of exponential decay was used to calculate the curves' slopes and the statistical difference between slopes.

Differences in the average pH during 10 min intervals (5 min before and 5 min after the time points) among CTR and OTC and the selected time points (i.e., 0, 4, 5, 6, and 7 h) were investigated by the analysis of variance (ANOVA) and the post hoc Tukey–Kramer test ($P < 0.05$), using the software SPSS Statistics (v. 21.0; IBM Corp., Armonk, NY, USA).

Lactic acid concentration (LAC), and the Log IBC mL^{-1}, the number of log cfu mL^{-1}, and the genes abundances were investigated by the Student-T test, using SPSS Statistics. The relationship among pH, LAC, and IBC evolution was investigated by the Pearson's correlation, using SPSS Statistic.

3. Results

3.1. Acidification curves

The pH evolution was continuously monitored and recorded, and acidification curves of CTR and OTC were presented in Fig. 1. Curve trends were quite different between CTR and OTC, with significantly ($P < 0.0001$) different slopes. pH values were statistically compared at the time points 0, 4, 5, 6, and 7 h, to evaluate the possible OTC effect on the starter culture acidification performance (Table 1). Statistical differences among the time points were always found in CTR, while no significant pH decrease was observed in OTC between the 4th and the 5th h and between 6th and the 7th h, confirming the acidification delay shown in Fig. 1. Statistical differences ($P < 0.05$) between CTR and OTC were observed after 5 h from inoculation.

Moreover, the time required to reach pH 5.6, a technological parameter used for syneresis and acidification monitoring in cheese-making, was also investigated (Cabizza et al., 2017, 2018). CTR and OTC reached the pH set after 426 and 788 min (about 7 and 13 h), respectively. Thus, the spiked sample showed a significant ($P < 0.001$) delay of 362 min (about 6 h) compared with the CTR.

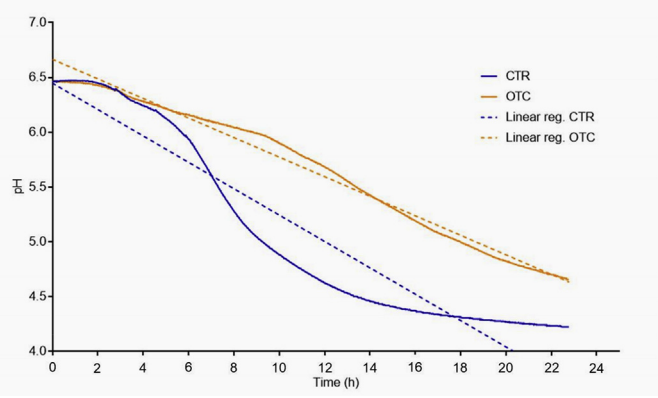

Fig. 1. Acidification curves (solid lines) of the microbial starter performed in control (CTR) and oxytetracycline-spiked (OTC) ovine milk. Curve equations of linear regression (dashed lines) of exponential decay are $y = (-0.002006 \times x) + 6.448$ and $y = (-0.001487 \times x) + 6.666$, respectively, and 1/slopes are −498.6 and −672.6, respectively ($P = 0.0001$).

Table 1
Average pH values in control (CTR) and oxytetracycline-spiked (OTC) ovine milk samples measured at the selected time points.[a]

Time (h)	CTR	OTC
0	6.47 ± 0.00^{f}	6.46 ± 0.00^{f}
4	6.24 ± 0.00^{e}	6.28 ± 0.00^{e}
5	6.13 ± 0.01^{cd}	6.22 ± 0.00^{de}
6	5.94 ± 0.01^{b}	6.16 ± 0.00^{c}
7	5.61 ± 0.02^{a}	6.10 ± 0.00^{c}

[a] Different superscript letters indicate statistically significant differences among pH values ($P < 0.05$), according to the Tukey's HSD post-hoc test.

3.2. Lactic acid determination

Lactic acid production was monitored at the time points set (0, 4, 5, 6, and 7 h), both in CTR and OTC samples (Fig. 2), to investigate possible delay in milk acidification due to OTC. Lactic acid concentration (LAC) at 0 h was below the LOD, both in CTR and OTC. Different production of lactic acid was observed between CTR and OTC, LAC was always lower in OTC, compared with CTR, but statistically significant ($P < 0.05$) differences were observed after 6 and 7 h of incubation.

3.3. Viable bacteria counts

Plate counts performed in M17 and MRS revealed not significant ($P < 0.05$) differences between CTR and OTC at all the time points tested (Fig. 3). Microbial growth was rapid in the first 4 h of incubation where thermophilic cocci, inoculated at 5.7–5.8 log cfu mL^{-1} both in CTR and OTC, reached 7.0 log cfu mL^{-1}. During the next 3 h, the bacterial growth kinetics was slower and, after 7 h incubation, thermophilic cocci reached 7.7 and 7.6 log cfu mL^{-1} in CTR and OTC, respectively.

Thermophilic lactobacilli, starting from an inoculum of 4.1–4.0 log cfu mL^{-1} in CTR and OTC, showed a lower increment (0.5 log), compared with cocci, during the first 4 h incubation, reaching a final concentration of 5.6–5.8 log cfu mL^{-1} after 7 h incubation (Fig. 3).

3.4. Individual bacterial counts

The OTC effect on the whole microbial community in ovine milk was investigated with the BactoScan™ method that relates the number of the single bacterial cells with the number of impulses. The log of individual bacterial counts (log IBC) was used to compare the OTC effect on the total bacteria number during the incubation.

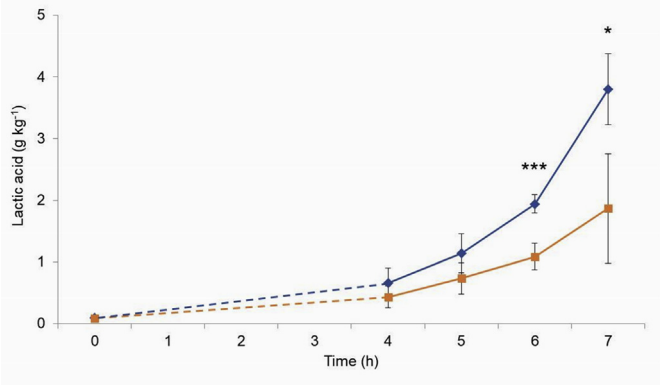

Fig. 2. Lactic acid production in control (CTR; ◆) and oxytetracycline-spiked (OTC; ■) ovine milk, immediately after inoculation (0 h), and after 4, 5, 6, and 7 h after inoculation. Student-T test was used to evaluate statistically significant differences in lactic acid production, at each time point, between CTR and OTC. *, $P < 0.05$; ***, $P < 0.001$.

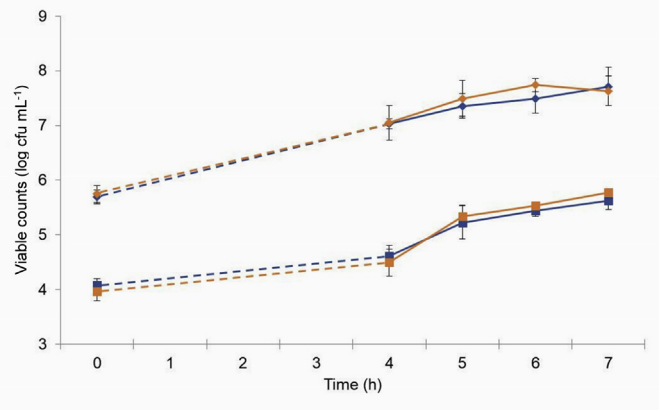

Fig. 3. Viable counts of the starter culture inoculated in control (CTR; blue symbols) and oxytetracycline-spiked (OTC; orange symbols) ovine milk, and performed in MRS (■) and M17 (◆), immediately after inoculation (0 h), and after 4, 5, 6, and 7 h after inoculation. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

The log IBC at the inoculum was about 3.7 and, during the first 4 h incubation, rose by 1.1 and 0.7 log IBC in the CTR and OTC, respectively (Fig. 4). Although the IBC increment was higher in CTR than OTC, no significant ($P < 0.05$) differences were found until 5 h incubation. Indeed, during the first 5 h the log IBC of CTR increased by 1.3 times that of the OTC, whereas from the 5th to the 7th hour of incubation the CTR log IBC increased by four times that of the OTC, making the average CTR and OTC log IBC significantly different at 6 ($P < 0.05$) and 7 h ($P < 0.01$) time points.

3.5. Correlation

Statistical correlation among milk acidification (pH), IBC, and lactic acid concentration (LAC), was performed (Table 2). Reduction in pH was always negatively correlated to IBC and LAC increase. In the CTR, all correlations were very close to 1, in absolute value, and always statistically significant. In OTC, correlations were, in absolute value, lower than those calculated for the CTR, and no significant correlation was calculated between LAC and IBC.

3.6. Quantitative real time PCR

Molecular analysis was used to evaluate the antibiotic effect on *S. thermophilus*, *Lb. helveticus*, and *Lb. delbrueckii* subsp. *lactis*, constituting the CHOOZIT® Su Casu LYO starter culture, by the amplification and quantification of species-specific genes.

The *lacZ* gene, coding for beta-galactosidase, was used to estimate the OTC effect on *S. thermophilus* by qPCR. Fig. 5A showed no significant ($P < 0.05$) differences in the inoculum (about 5.2 log cfu mL^{-1}) at the time-point 0 h, investigated by the Student-T test. However, delayed *S. thermophilus* growth was observed and significant differences between CTR and OTC were found at 4, 5 and 6 h of incubation, but not after 7 h. During the first 4 h of incubation, the *lacZ* gene abundance reached 7.4 log cfu mL^{-1} in the CTR. Conversely, the increment of this gene in OTC-spiked milk was progressively distributed throughout the 7 h of incubation, not

Table 2
Statistical correlation of pH, individual bacterial counts (IBC), and lactic acid concentration (LAC) in control (CTR) and oxytetracycline-spiked (OTC) ovine milk samples.

Treatment	Variable	Variable	Correlation	*p*
CTR	pH	LAC	−0.997	0.003
	pH	IBC	−0.991	0.009
	LAC	IBC	0.979	0.021
OTC	pH	LAC	−0.971	0.029
	pH	IBC	−0.974	0.026
	LAC	IBC	0.900	0.100

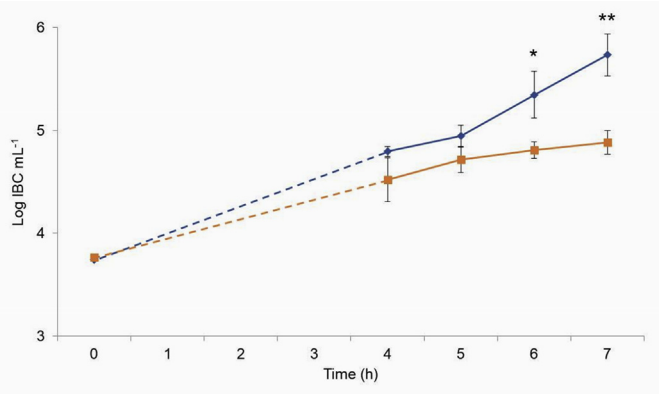

Fig. 4. Individual bacterial counts (IBC) in control (CTR; ◆) and oxytetracycline-spiked (OTC; ■) ovine milk, immediately after inoculation (0 h), and 4, 5, 6, and 7 h after inoculation. Student-T test was used to evaluate statistically significant differences in IBC, at each time point, between CTR and OTC. *, $P < 0.05$; **, $P < 0.01$.

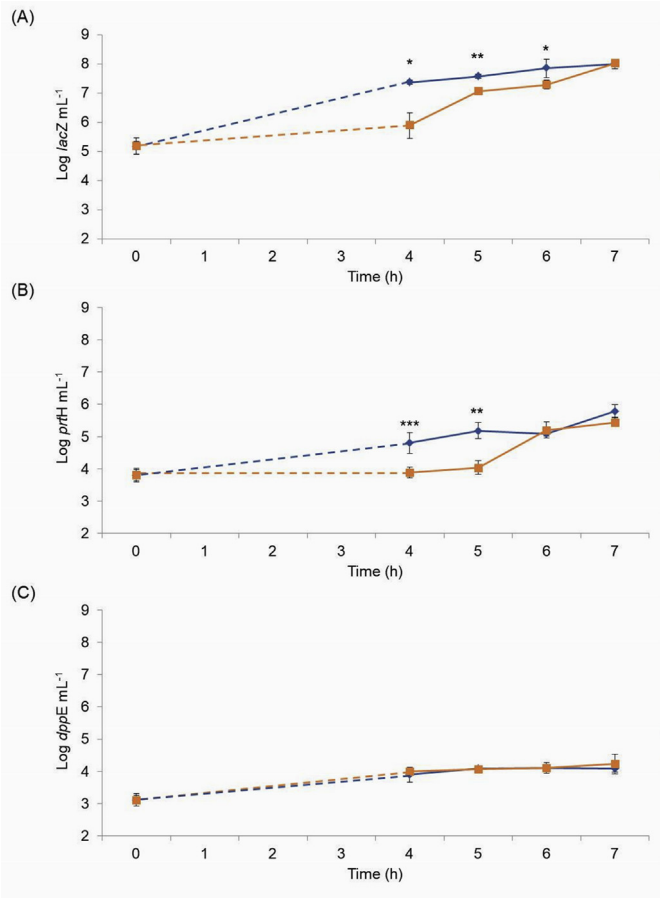

Fig. 5. Quantification of the species-specific genes *lacZ* for *S. thermophilus* (A), *prt*H for *Lb. helveticus* (B), and *dpp*E for *Lb. delbrueckii* subsp. *lactis* (C) by qPCR in control (CTR; ◆) and oxytetracycline-spiked (OTC; ■) ovine milk, immediately after inoculation (0 h), and after 4, 5, 6, and 7 h after inoculation. Student-T test was used to evaluate statistically significant differences in genes abundances, at each time point, between CTR and OTC. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

reaching a level comparable with the CTR (7.3 log cfu mL^{-1}) until after 6 h. This means that *S. thermophilus* development in OTC-spiked milk was 2 h late, compared with the CTR. Indeed, from the 4th to the 6th h a significant difference in the abundance of *lac*Z between CTR and OTC was observed.

The OTC effect on *L. helveticus* was estimated by the quantification of *prt*H gene, coding for cell-enveloped associated proteinase synthesis. Similar to observations for *lac*Z, for *prt*H the level of the inoculum was the same for both the CTR and OTC (3.8 log cfu mL^{-1}) (Fig. 5B). Significant differences between CTR and OTC were observed at 4 and 5 h, whereas after 6 and 7 h of incubation the *prt*H abundance between the two treatments was not different. In OTC, as observed for *S. thermophilus*, *Lb. helveticus* showed a delay of about 2 h in reaching a level of growth similar to the CTR.

The gene coding for dipeptide transport system production (*dpp*E) was used for the estimation of the *Lb. delbrueckii* subsp. *lactis* population. In Fig. 5C an increase from 3.1 to about 4.0 log cfu mL^{-1} in the abundance of *dpp*E gene after 4 h of incubation was shown for both CTR and OTC. However, during the next 3 h, the *dpp*E level was kept constant. Furthermore, unlike what was observed for the others two genes, no significant differences were found between CTR and OTC at all the time-points tested. No *Lb. delbrueckii* subsp. *lactis* development delay was detected in OTC samples compared with the CTR.

4. Discussion

The presence of antibiotic residues in milk can be related with problems mainly regarding consumer safety and technological aspects (Priyanka, Panigrahi, Singh Sheoran, & Ganguly, 2017). Several papers have demonstrated the propensity of tetracyclines in milk to concentrate through the cheese-making (Cabizza et al., 2018, 2017; Gajda, Nowacka-Kozak, Gbylik-Sikorska, & Posyniak, 2018). Recently, Gajda et al. (2018) studied the behaviour of tetracyclines (i.e., tetracycline, OTC, chlortetracycline, and doxycycline) in bovine raw milk spiked at different MRL levels. Tetracyclines were found in milk after a low-temperature-long-term (LTLT) thermal treatment (63 °C for 30 min), and the molecules were concentrated four to five times in cheese. A similar trend was observed by Cabizza et al. (2017; 2018) in ovine milk spiked with OTC at MRL level. They did not note degradation of the molecule after thermal treatment and reported a concentration of the molecule in 1-day cheese about 3.8 times higher than that in milk. This concentration could potentially lead to a not negligible risk for human health.

Moreover, in the same study, OTC in milk affected the technological performance of microbial starters. Indeed, a dose-dependent delay during the acidification phase was observed both for raw and thermised milk. The authors suggested a partial inhibition caused by the molecule against the commercial starter composed of *Lb. helveticus, Lb. delbrueckii* subsp. *lactis,* and *S. thermophilus*, despite the OTC concentration being much lower than the microbiological cut-off of 4 mg L^{-1} set by EFSA (Rychen et al., 2018) to discriminate between tetracyclines-sensitive and -resistant strains belonging to these species. However, 24 h after the inoculum, Cabizza et al. (2017, 2018), did not find significant differences in SLAB viable counts, hypothesising a temporary OTC effect on SLAB development, limited only to the early hours of acidification phase. These results raised the need to focus interest on the early acidification period.

The present work is a zoom on the SLAB activity in OTC spiked ovine milk, during the first seven hours from inoculation, to better investigate the antibiotic effect using a multi-technical approach. At each sampling point, the results confirmed a protracted delay in acidification of the OTC spiked milk compared with the CTR, always associated with no significant differences in viable microbial counts. However, it should be taken into account that, although plate counts have the advantage to allow discrimination between dead and alive cells, this technique has the limit of expressing the results as number of colony forming units. Since each colony can be originated from one to many cells, this way to report microbiological data, though universally used, can lead to miscounting (undercounting) the real number of singular bacterial cells present in a sample (Chessa, Paba, Daga, & Comunian, 2018; Hazan, Que, Maura, & Rahme, 2012; Sutton, 2011). Particularly, it happens when bacteria grow in long chains (e.g., *S. thermophilus*) or clumps (e.g., *Staphylococcus*) (Lee, 2008). Hence, even if the same number of cfu mL^{-1} is present in both the OTC and CTR samples, a different number of bacterial cells could be contained in the two samples, producing different amounts of lactic acid.

To overcome this drawback of the method, other two techniques, i.e., IBC by BactoScan™ and real-time qPCR, were used to determine the number of single bacterial cells.

IBC revealed significant differences between CTR and OTC, but only at 6 and 7 h from inoculation, confirming the hypothesis that a higher number of bacteria were growing and acidifying the milk in the CTR than in OTC. This is corroborated by the positive and significant correlation between IBC increase and LAC in the CTR, not statistically significant in OTC. Increase of lactic acid production, especially after 6 and 7 h of incubation, reflected the different SLAB community development in the CTR, compared with OTC. The antibiotic probably limited the growth of bacteria responsible for lactic acid production and, consequently, milk acidification. However, this technique does not allow discrimination among bacterial species, and understanding which are more affected by the antibiotic, even if it is conceivable that *S. thermophilus*, well-known to be responsible of the early phases of acidification (Beal & Corrieu, 1991; Courtin, Monnet, & Rul, 2002; Ma et al., 2015), should be the species most influenced.

The microbial SLAB population was more deeply investigated by real-time qPCR, which allowed to perform a species-specific monitoring of the three starter strains *S. thermophilus*, *Lb. helveticus*, and *Lb. delbrueckii* subsp. *lactis*. According to the acidification curves, which showed a significant difference between the CTR and OTC already after 4 h of incubation, the abundance of *lac*Z copy number suggested a faster development rate (from the 4th to the 6th h) of *S. thermophilus*, compared with the two *Lactobacillus* strains. Indeed, *Lb. helveticus prt*H gene abundance seemed to tend increasing in a longer period (from the 4th to the 7th h), while *Lb. delbrueckii* subsp. *lactis dpp*E gene showed a flat trend. Therefore, qPCR analysis confirmed that the delay observed in OTC could be attributable principally to *S. thermophilus*, which turned out to be the most important species in contributing to the acidification activity during the first hours of the process. This is corroborated by the fact that it constitutes the main portion of the starter microbiota throughout the whole period monitored. Therefore, the poor SLAB acidification performance (i.e., lower lactic acid production) in OTC-spiked milk samples, which Cabizza et al. (2017, 2018) assumed could be attributed to a change in their metabolism, was actually due to a lower concentration of *S. thermophilus*, compared with the CTR. For this reason, this species seemed to be more affected by the antibiotic residues, as previously reported by Cogan (1972), while *Lb. helveticus* was affected for a shorter time, and *Lb. delbrueckii* subsp. *lactis* was not affected at all.

5. Conclusions

The trials carried out in the present study confirmed a significant acidification delay in ovine milk that was thermised after spiking with OTC at MRL level. The multi-technical approach used

allowed to shed light on the reason of this delay, already detectable after 4 h and lasting at least 2–3 h, that was mainly due to a late SLAB development, particularly *S. thermophilus*, with a consequent lower lactic acid production.

Thus, the OTC residues amount in milk allowed by the European Commission should be reduced since their concentration increase in cheese, exceeding the MRL stated for milk, with possible risks for human health. Furthermore, OTC, negatively affecting the early phases of milk fermentation, leads to an insufficient acidification, which could not be able to counteract the development of spoilage/pathogen bacteria, thus resulting in safety problems (short-ripening products), cheese defects, and economic losses.

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References

Aalipour, F., Mirlohi, M., Jalali, M., & Azadbakht, L. (2015). Dietary exposure to tetracycline residues through milk consumption in Iran. *Journal of Environmental Health Science and Engineering, 13*, 80–87.

Beal, C., & Corrieu, G. (1991). Influence of pH, temperature, and inoculum composition on mixed cultures of *Streptococcus thermophilus* 404 and *Lactobacillus bulgaricus* 398. *Biotechnology and Bioengineering, 38*, 90–98.

Beltrán, M. C., Morari-Pirlog, A., Quintanilla, P., Escriche, I., & Molina, M. P. (2018). Influence of enrofloxacin on the coagulation time and the quality parameters of goat's milk yoghurt. *International Journal of Dairy Technology, 71*, 105–111.

Berruga, M. I., Beltrán, M. C., Novés, B., Molina, A., & Molina, M. P. (2012). Effect of penicillins on the acidification of yogurt made from Ewe's milk during the storage. In A. Mendez-Vilas (Ed.), *Science and technology against microbial pathogens. Proceedings of the international conference on antimicrobial Research (ICAR2010), valladolid, Spain, July 2011* (pp. 145–149). London, UK: World Scientific.

Berruga, M. I., Molina, A., Althaus, R. L., & Molina, M. P. (2016). Control and prevention of antibiotic residues and contaminants in sheep and goat's milk. *Small Ruminant Research, 142*, 38–43.

Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., et al. (2017). Transfer of oxytetracycline from ovine spiked milk to whey and cheese. *International Dairy Journal, 70*, 12–17.

Cabizza, R., Rubattu, N., Salis, S., Pes, M., Comunian, R., Paba, A., et al. (2018). Impact of a thermisation treatment on oxytetracycline spiked ovine milk: Fate of the molecule and technological implications. *LWT, 96*, 236–243.

Chang, Q., Wang, W., Regev-Yochay, G., Lipsitch, M., & Hanage, W. P. (2015). Antibiotics in agriculture and the risk to human health: How worried should we be? *Evolutionary Applications, 8*, 240–247.

Chessa, L., Paba, A., Daga, E., & Comunian, R. (2018). Effect of growth media on natural starter culture composition and performance evaluated with a polyphasic approach. *International Journal of Dairy Technology, 72*, 152–158.

Chopra, I., & Roberts, M. (2001). Tetracycline antibiotics: Mode of action, applications, molecular biology, and epidemiology of bacterial resistance. *Microbiology and Molecular Biology Reviews, 65*, 232–260.

Cogan, T. M. (1972). Susceptibility of cheese and yoghurt starter bacteria to antibiotics. *Applied Microbiology, 23*, 960–965.

Courtin, P., Monnet, V., & Rul, F. (2002). Cell-wall proteinases PrtS and PrtB have a different role in *Streptococcus thermophilus/Lactobacillus bulgaricus* mixed cultures in milk. *Microbiology, 148*, 3413–3421.

Cremonesi, P., Vanoni, L., Morandi, S., Silvetti, T., Castiglioni, B., & Brasca, M. (2011). Development of a pentaplex PCR assay for the simultaneous detection of *Streptococcus thermophilus, Lactobacillus delbrueckii* subsp. *bulgaricus, L. delbrueckii* subsp. *lactis, L. helveticus, L. fermentum* in whey starter for Grana Padano cheese. *International Journal of Food Microbiology, 146*, 207–211.

EC. (2010). *Annex to Commission Regulation (EU) No. 37/2010 on pharmacologically active substances and their classification regarding maximum residue limits in foodstuffs of animal origin*. Brussels, Belgium: European Commission.

EMA. (2016). *Sales of veterinary antimicrobial agents in 29 European countries in 2014. Trends from 2011 to 2014*. London, UK: European Medicines Agency. Sixth ESVAC report (EMA/61769/2016).

Gajda, A., Nowacka-Kozak, E., Gbylik-Sikorska, M., & Posyniak, A. (2018). Tetracycline antibiotics transfer from contaminated milk to dairy products and the effect of the skimming step and pasteurisation process on residue concentrations. *Food Additives & Contaminants: Part A, 35*, 66–76.

Hazan, R., Que, Y. A., Maura, D., & Rahme, L. G. (2012). A method for high throughput determination of viable bacteria cell counts in 96-well plates. *BMC Microbiology, 12*. Article 259.

Heuer, H., Schmitt, H., & Smalla, K. (2011). Antibiotic resistance gene spread due to manure application on agricultural fields. *Current Opinion in Microbiology, 14*, 236–243.

Hubaux, A., & Vos, G. (1970). Decision and detection limits for linear calibration curves. *Analytical Chemistry, 42*, 849–855.

ISO. (2017). *General requirements for the competence of testing and calibration laboratories*. Geneva, Switzerland: International Standardisation Organisation. ISO/IEC 17025:2017.

Khosrokhavar, R., Hosseini, M. J., Amini, M., Pirali-Hamedani, M., Ghazi-Khansari, M., & Bakhtiarian, A. (2008). Validation of an analytical methodology for determination of oxytetracycline residue in milk by HPLC with UV detection. *Toxicology Mechanisms and Methods, 18*, 351–354.

Lee, P. S. (2008). Quantitation of microorganisms. In E. Goldman, & L. H. Green (Eds.), *Practical handbook of microbiology* (2nd ed., pp. 11–29). Boca Raton, FL, USA: CRC Press, Taylor and Francis Group.

Ma, C., Ma, A., Gong, G., Liu, Z., Wu, Z., Guo, B., et al. (2015). Cracking *Streptococcus thermophilus* to stimulate the growth of the probiotic *Lactobacillus casei* in co-culture. *International Journal of Food Microbiology, 210*, 42–46.

Mathur, S., & Singh, R. (2005). Antibiotic resistance in food lactic acid bacteria-a review. *International Journal of Food Microbiology, 105*, 281–295.

Naik, L., Sharma, R., Mann, B., Lata, K., Rajput, Y. S., & Surendra Nath, B. (2017). Rapid screening test for detection of oxytetracycline residues in milk using lateral flow assay. *Food Chemistry, 219*, 85–92.

Priyanka, Panigrahi, S., Singh Sheoran, M., & Ganguly, S. (2017). Antibiotic residues in milk- a serious public health hazard. *Journal of Environmental Life Sciences, 2*, 99–102.

Roca, I., Akova, M., Baquero, F., Carlet, J., Cavaleri, M., Coenen, S., et al. (2015). The global threat of antimicrobial resistance: Science for intervention. *New Microbes New Infections, 6*, 22–29.

Rychen, G., Aquilina, G., Azimonti, G., Bampidis, V., Bastos, M. d. L., Bories, G., et al. (2018). Guidance on the characterisation of microorganisms used as feed additives or as production organisms. *EFSA Journal, 16*, e05206.

Smith, D. L., Dushoff, J., & Morris, J. G. (2005). Agricultural antibiotics and human health. *PLoS Medicine, 2*. Article e232.

Sutton, S. (2011). Accuracy of plate counts. *Journal of Validation Technology, 17*, 42–46.

Tona, G. O., & Olusola, A. D. (2014). Determination of tetracycline antibiotic residues in dairy products sold in Ogbomoso, South-Western Nigeria. *International Journal of Food, Agriculture and Veterinary Sciences, 4*, 136–140.

Willis, C. (2000). Antibiotics in the food chain: Their impact on the consumer. *Reviews in Medical Microbiology, 11*, 153–160.