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RESEARCH ARTICLE

Legally admissible amounts of antibiotics in milk affect the growth of lactic acid bacteria

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The antibiotic administration in dairy livestock is a current widespread practice. The purpose of the study was to evaluate whether milk from antibiotic-treated animals, following the application of the withdrawal time and fully complying with European requirements in relation to residue content, affected the development of lactic acid bacteria. Forty-five raw milk samples were collected after the withdrawal period and analysed to verify their compliance with the European maximum limits using four commercial screening tests. Lactobacillus delbrueckii *subsp.* bulgaricus *growth and acidifying activity were delayed from 5 to 8 h in milk from cow treated with β-lactams and sulfonamides present in low concentration (cephalosporins < 15 μg/kg; penicillins < 8 μg/kg; sulfonamides < 10 μg/kg) and their effect persisted over time. No influences were detected in* Streptococcus thermophilus *and* Lb. helveticus *development. The use of antibiotics can hamper the starter performances, opening questions about the safety of dairy products and human health.*

Keywords Antibiotic, Withdrawal period, β-lactams, Sulfonamides, Lactic acid bacteria, Raw milk biodiversity.

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INTRODUCTION

Antibiotics are naturally, semi-synthetic or synthetic compounds with antimicrobial activity that can be applied parenterally, orally or topically. These substances are widely used in the livestock care to cure infections such as mastitis, respiratory and foot diseases. In addition, cow antibiotic therapy is also used to prevent intramammary pathologies in the dry period (McEwen and Fedorka-Cray 2002). The trend in antibiotic use in dairy farming has shown three distinct phases over the past decade. Initially, there was an increase in antibiotic use, followed by a stabilisation period, and then a subsequent decrease coinciding with increasing societal concerns about antibiotic use. Nevertheless, the antibiotic market in dairy cattle is expected to reach $2046.0 million by 2025, and $2705.0 million by 2030 (Paramasivam *et al*. 2023).

Tetracyclines, β-lactams, quinolones, sulfonamides, aminoglycosides, cephalosporins and chloramphenicol are the most frequently used veterinary drugs in dairy cattle (Chiesa *et al*. 2020). Once administered to the animal, a big part of the antibiotic is metabolised and later excreted by urine or faeces. However, a portion of the drug may persist in the animal and can be found in the milk. In particular, in the mastitis treatment process, the antibiotics can be easily transferred from the mammary gland to the milk reducing its quality and safety (Sachi *et al*. 2019). For this reason, when a cow is treated with antibiotics, its milk is generally separated from the bulk tank for a specific period called 'withdrawal period'. The period is considered as the minimum time between the last administration of a veterinary medicinal product to an animal and the production of foodstuffs from that animal (EU 2019). The length of this period is linked to the antibiotic characteristics,

and it varies according to active substances, pharmaceutical form and strength (Schmerold *et al*. 2023). This precaution is necessary to avoid potential risks on the human health and the spread of antibiotic resistances related to the consumption of dairy products (Rajala-Schultz *et al*. 2021). Moreover, to protect public health, the European Union set maximum residue limits (MRLs) for pharmacologically active substances in milk and animal-origin foods (EU 2010).

In addition to human health, the antibiotic residues contained in milk could negatively affect the cheese-making process by hampering the starter culture growth, interfering with the milk coagulation and causing improper cheese ripening (Quintanilla *et al*. 2018). Moreover, an insufficient pH lowering can lead to an early fermentation supported by clostridia and other spoilage microorganisms and consequent defects in the sensory characteristics of yogurt and cheeses (Pecorari *et al*. 2003). The impact of antibiotic residues on the starter culture development was evaluated by growing lactic acid bacteria (LAB) in milk spiked with known amounts of drugs. These studies showed that the acidification capability of *Lactococcus lactis*, *Lactobacillus delbrueckii*, *Lb. helveticus* and *Streptococcus thermophilus* was considerably affected by the presence of the antibiotics tested (fluoroquinolones, lincosamides, macrolides, penicillins and tetracyclines) (Paba *et al*. 2019; Quintanilla *et al*. 2019; Chiesa *et al*. 2020). Moreover, Navrátilova *et al*. (2022) displayed that the ceftiofur (cephalosporin) spiked in milk at the MRL level caused an inhibition of the metabolic activity of five different yogurt cultures. On the contrary, Beltrán *et al*. (2018, 2023) highlighted that the legally admissible amounts of enrofloxacin and ciprofloxacin did not influence the coagulation time and properties of yogurt and cheese from goat milk. Nevertheless, few data are available on the LAB development in real milk samples collected from cows treated with different classes of veterinary drugs (Berruga *et al*. 2008; Quintanilla *et al*. 2018, 2021).

Therefore, the aim of this study was to evaluate the LAB growth and acidifying activity in cow milk collected at the end of the withdrawal period and 2 days later. For this purpose, we considered 45 raw milk samples collected from 23 cows treated with two different classes of antibiotics.

MATERIALS AND METHODS

Experimental procedure and sample preparation

Forty-five raw milk samples (1 L of each sample) were collected from 23 different cows treated with drugs containing antibiotics belonging to β-lactams and sulfonamides classes (Figure 1; Table 1). The animals were chosen from farms that supply milk to three different dairies producing Grana Padano cheese and located in three different Lombardy provinces (Bergamo, Lodi and Mantova). After milking, all samples were immediately frozen and kept at –18°C. To evaluate the antibiotic's impact on the bacterial development, milk samples were collected from the same cow at the end of the withdrawal period (n. 22, sample A) and 2 days later (n. 23, sample B). In each farm, the presence of antibiotic residues was analysed using three different commercial screening tests: Delvotest SP NT (Delvotest; DSM Food Specialties, Delft, the Netherlands), Charm CowSide II (Charm Sciences Inc., Lawrence, MA, USA) and CMT Copan Milk Test (Copan Italia S.p.a., Brescia, Italy). Tests were carried out according to the instructions of the manufacturers. Before the analysis, all samples were thawed in a refrigerator and then the milk aliquots were analysed to verify the compliance with MRLs for the presence of β-lactams and sulfonamides using the 4Sensor BSTQ assay (Unisensor, Seraing, Belgium). Later, 700 mL of each sample was heated to 63°C in a water bath and then cooled to 37°C, in cold water as previously described by Paba *et al*. (2019). At the end of the heat treatment, the samples were inoculated with the LAB cultures.

Next, to simulate what happens when milk from a treated animal is added to the bulk milk, 100 mL of each raw milk sample was diluted in UHT whole milk (1:50 vol/vol) purchased from a local supermarket (samples 50A and 50B). After dilution, these samples were thermised as described above and then inoculated with LAB strains (Figure 1).

Bacterial strains

Three LAB species commonly used in dairy fermentation were considered in this study. *Streptococcus thermophilus* ST47 and *Lactobacillus delbrueckii* subsp. *bulgaricus* LB08 were previously isolated from commercial yogurt purchased from the Italian market, while *Lb. helveticus* DSM 20075 was provided by the Deutsche Sammlung von Mikroorganismen und Zellkulturen (Braunschweig, Germany). Before testing, the strains were grown twice in sterile reconstituted skim milk (10% w/v) (Sacco S.r.l., Cadorago, Italy) and incubated aerobically overnight at 37°C. All cultures were preserved in litmus milk (Biolife Italiana, Milan, Italy) at −18°C.

Determination of minimal inhibitory concentrations (MICs)

The MICs of *St. thermophilus*, *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* strains were determined by applying MIC test strips (Liofilchem, Roseto degli Abruzzi, Italy) on ISO-Sensitest agar medium (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Briefly, saline suspensions with concentration of ~10^8 cells/mL were obtained from overnight cultures. A sterile cotton swab was dipped in each cell suspension and used to inoculate the ISO-Sensitest agar plates by swabbing in three directions. After drying the surface, the gradient strips were placed on the agar plates and incubated

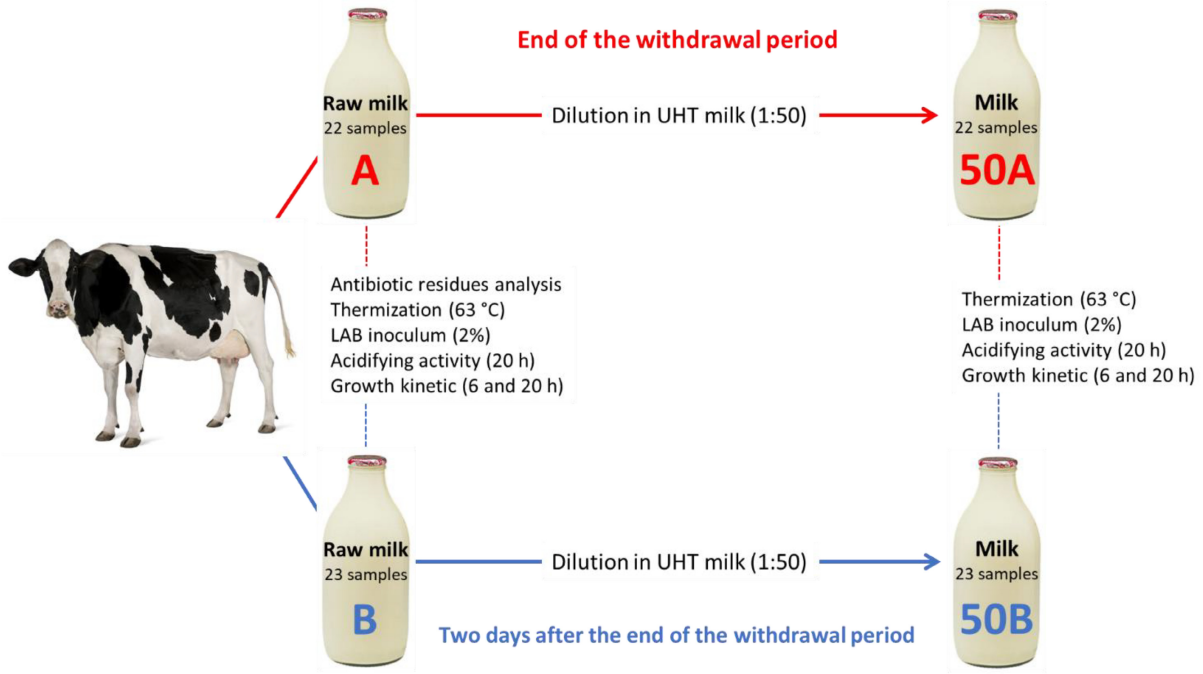

Figure 1 Summary of the experimental scheme used in this study.

Table 1 Classes of antibiotics and milk samples considered in the study.

			Milk samples				
Class of antibiotic	Sub-class	Compound	A	B	50A	50B	Total
β-lactams	Cephalosporins	Ceftiofur	7	8	7	8	30
β-lactams	Cephalosporins	Cefalonium	1	1	1	1	4
β-lactams	Penicillins	Ampicillin and Dicloxacillin	5	5	5	5	20
β-lactams	Penicillins + Clavulanic acid	Amoxicillin and Clavulanic acid	5	5	5	5	20
Sulfonamides		Sulfadiazine and Trimethoprim	4	4	4	4	16
Total			22	23	22	23	90

50A and 50B, samples A and B diluted 1:50 vol/vol in UHT milk; A, samples collected at the end of the withdrawal period; B, samples collected at 2 days after the end of the withdrawal period.

aerobically at 37°C per 24 h. The MIC was defined by the intersection between the test stripe and the edge of the bacterial growth ellipse. The antibiotics tested were ampicillin, chloramphenicol, clindamycin, erythromycin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin according to the requirements of the European Food Safety Authority guidance document for the microorganisms intentionally used in the food chain (EFSA 2018).

LAB inoculum and acidification activity

To evaluate the acidifying activity of LAB strains in milk samples, a multi-channel pH meter (Acidification Monitoring System and Analyser, Star Ecotronics, Milan, Italy) was used. Overnight cultures were inoculated at a level of 2% (vol/vol) in 100 mL of milk sample to obtain a final concentration of 6.0–7.0 $\log_{10}$ cfu/mL. Inoculated milk samples were incubated at 37°C in a controlled water bath for 20 h. Combined pH electrodes (In Lab Power 51 343 110, Mettler-Toledo, Greifensee, Switzerland) were calibrated using standard solutions with pH 4.0 and 7.0 (Scharlab, Barcelona, Spain) before each analysis. During fermentation, pH was automatically recorded at 10-min intervals. From the collected data, the following kinetic parameters were calculated: (1) maximum acidification rate (V_m; pH unit/h), (2) time to reach V_m (T_m, h), (3) ΔpH ($pH_{zero\ time} - pH_{at\ time}$) after 6 and 20 h of incubation, (4) the pH value achieved at the end of the trial (pH_{min}) and the time required to reduce the pH value by one unit ($T_{\Delta pH}$) (Morandi and Brasca 2012; Morandi *et al.* 2022). In each

Table 2 Minimum inhibitory concentrations (MIC) of antibiotics towards the LAB strains (mg/L).

Antibiotic	*Streptococcus thermophilus* ST47		*Lactobacillus bulgaricus* LB08		*Lactobacillus helveticus* DSM 20075	
	Cut-off[a]	MIC	Cut-off[a]	MIC	Cut-off[a]	MIC
Ampicillin	2	0.023	2	0.016	2	0.125
Chloramphenicol	4	3	4	<0.016	4	3
Clindamycin	2	0.016	4	0.125	4	0.25
Erythromycin	2	0.064	1	0.032	1	0.125
Gentamicin	32	0.75	16	16	16	4
Kanamycin	n.r.	–	16	>256	16	8
Streptomycin	64	32	16	48	16	0.38
Tetracycline	4	0.023	4	0.047	4	2
Vancomycin	4	0.25	2	0.125	2	0.75

MIC, minimal inhibitory concentrations; n.r., not required.
[a]Cut-off values indicated by the EFSA guidance document (EFSA 2018).

experiment, the control (CTRL) consisted of UHT whole milk inoculated with the LAB strains. All measurements were made in duplicate.

LAB content

Bacterial counts were performed at the beginning of the incubation and during the fermentation process after 6 and 20 h. *St. thermophilus* was enumerated on M17 agar with 0.5% (w/v) lactose (Biolife Italiana), whereas *Lb. delbrueckii* subsp. *bulgaricus* and *Lb. helveticus* were counted on MRS agar at pH 5.4 (Biolife Italiana) under anaerobic conditions (Anaerocult A, Merck). Both media were incubated at 37°C for 48 and 72 h. All determinations were made in duplicate.

Statistical analysis

Data were analysed using SAS (version 9.4; SAS Institute Inc., Cary, NC, USA). The results obtained for plate counts were transformed to log10 for statistical analysis to obtain a normal distribution of the residuals in linear statistical models. A general linear model (GLM) analysis was performed to evaluate unbalanced data on the antibiotic susceptibility of LAB strains and the growth and acidification activity of LAB strains, using a simple model with fixed effects of type of antibiotic, time of milk collection and dilution on UHT milk.

RESULTS

In the present study, we have evaluated the LAB development in milk from cows treated with different veterinary drugs containing antibiotics belonging to two different classes: β-lactams (cephalosporins, penicillins and penicillins + clavulanic acid) and sulfonamides. Among the β-lactams, a high number of samples (15 out of 37) came from cows cured with ceftiofur, a third-generation cephalosporin with a zero-withdrawal period (Table 1).

Antibiotic susceptibility

Prior to starting the experiments, the antibiotic susceptibility of LAB strains was evaluated considering the microbiological cut-off values indicated by EFSA guidelines (EFSA 2018). *St. thermophilus* ST47 and *Lb. helveticus* DSM 20075 resulted in susceptible to all the tested antibiotics (Table 2). *Lb. delbrueckii* subsp. *bulgaricus* LB08 showed intrinsic resistances to kanamycin (MIC 16 mg/L) and streptomycin (MIC > 256 mg/L) (Zarzecka *et al*. 2020). These results did not affect the trials since the aminoglycosides (kanamycin and streptomycin) are not part of the agents considered in this study (Table 2).

Antibiotic residue detection in raw milk samples

Forty-five raw milk samples from the 23 different cows were analysed prior to use by the microbial inhibitor test 4Sensor BSTQ. Interesting to notice that the cephalosporins and sulfonamides detection limits of this test are much lower than the MRLs considered. No positive results were observed (Table 3), confirming that the concentrations of these compounds were below the MRLs established by European regulation (EU 2010). The same results were obtained at the farm level.

Growth and acidification activity of LAB strains

The first data analysis was performed considering all the findings related to bacterial counts and acidification parameters without taking into account the different antibiotics used for the cow treatments. In case differences in the strains' activity between CTRL samples and raw milk were

Table 3 Presence of antibiotic residues in raw milk samples by using 4Sensor BSTQ kit.

					Milk samples			
					A		B	
Class of antibiotic	Sub-class	Compound	MRL (μg/kg)[a]	4Sensor BSTQ (μg/kg)	n	Positive	n	Positive
β-lactams	Cephalosporins	Ceftiofur	100	10–15	7	0	8	0
		Cefalonium	20	3–5	1	0	1	0
	Penicillins	Ampicillin	4	3–4	5	0	5	0
		Dicloxacillin	30	6–8				
	Penicillins +	Amoxicillin	4	3–4	5	0	5	0
	Clavulanic acid	Clavulanic acid	no MRL	–				
Sulfonamides		Sulfadiazine	100	8–10	4	0	4	0
		Trimethoprim	100	–				

A, samples collected at the end of the withdrawal period; B, samples collected after 2 days of the end of the withdrawal period.
[a]Maximum Residue Limits (MRL) according to European regulation REG. 37/2010/CE. (EU 2010).

Table 4 Microbial counts and kinetic acidification parameters of starter cultures inoculated in milk samples.

				Milk sample			
Starter culture	Parameters	Time (h)	CTRL (n. 5)	A (n. 22)	B (n. 23)	50A (n. 22)	50B (n. 23)
Streptococcus thermophilus ST47	Bacterial count (log_{10} cfu/mL)	0	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.1	7.4 ± 0.1
		6	8.7 ± 0.4	8.6 ± 0.2	8.7 ± 0.2	8.8 ± 0.2	8.9 ± 0.2
		20	8.9 ± 0.3	8.7 ± 0.5	8.7 ± 0.4	8.7 ± 0.4	8.6 ± 0.7
	V_m (pH unit/h)		0.5 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.7 ± 0.2
	T_m (h)		4.5 ± 0.8	4.4 ± 0.9	4.3 ± 0.7	4.3 ± 0.7	4.3 ± 0.5
	ΔpH	6	1.3 ± 0.2	1.4 ± 0.3	1.4 ± 0.3	1.5 ± 0.3	1.5 ± 0.2
		20	2.1 ± 0.1	2.1 ± 0.2	2.1 ± 0.2	2.2 ± 0.1	2.2 ± 0.1
	pH_{min}	20	4.5 ± 0.8	4.4 ± 0.9	4.3 ± 0.7	4.3 ± 0.7	4.3 ± 0.5
Lactobacillus helveticus DSM 20075	Bacterial count (log_{10} cfu/mL)	0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0
		6	8.2 ± 0.2	8.3 ± 0.5	8.2 ± 0.5	8.3 ± 0.5	8.3 ± 0.5
		20	9.1 ± 0.2	8.8 ± 0.2	8.8 ± 0.2	9.1 ± 0.2	9.0 ± 0.2
	V_m (pH unit/h)		0.2 ± 0.1	0.2 ± 0.3	0.2 ± 0.1	0.2 ± 0.2	0.2 ± 0.1
	T_m (h)		8.0 ± 2.3	7.9 ± 3.5	7.8 ± 1.7	8.1 ± 5.0	7.8 ± 3.9
	ΔpH	6	0.4 ± 0.1	0.4 ± 0.2	0.5 ± 0.3	0.4 ± 0.1	0.4 ± 0.2
		20	2.4 ± 0.2	2.2 ± 0.2	2.2 ± 0.2	2.3 ± 0.3	2.3 ± 0.3
	pH_{min}	20	4.0 ± 0.2	4.2 ± 0.2	4.2 ± 0.2	4.1 ± 0.3	4.1 ± 0.3
Lactobacillus delbrueckii subsp. *bulgaricus* LB08	Bacterial count (log_{10} cfu/mL)	0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0	7.3 ± 0.0
		6	8.5 ± 0.5	8.0 ± 0.4	7.9 ± 0.5	8.2 ± 0.5	8.1 ± 0.6
		20	8.9 ± 0.4	8.8 ± 0.5	8.9 ± 0.4	8.9 ± 0.4	9.0 ± 0.4
	V_m (pH unit/h)		0.5 ± 0.2^{B}	0.3 ± 0.1^{A}	0.3 ± 0.1^{A}	0.3 ± 0.1^{A}	0.3 ± 0.1^{A}
	T_m (h)		6.9 ± 4.3^{A}	12.7 ± 5.5^{B}	12.7 ± 4.7^{B}	11.1 ± 5.0^{B}	11.9 ± 4.9^{B}
	ΔpH	6	0.6 ± 0.4^{B}	0.3 ± 0.5^{A}	0.2 ± 0.3^{A}	0.4 ± 0.5^{A}	0.2 ± 0.4^{A}
		20	2.5 ± 0.0^{B}	1.6 ± 0.7^{A}	1.6 ± 0.7^{A}	1.8 ± 0.7^{A}	1.8 ± 0.7^{A}
	pH_{min}	20	3.9 ± 0.0^{A}	4.8 ± 0.7^{B}	4.8 ± 0.7^{B}	4.6 ± 0.7^{B}	4.8 ± 0.7^{B}

Data are expressed as means ± SD. ^{A,B}Means with different letters within a row are significantly different ($P < 0.05$).
50A and 50B, samples A and B diluted in UHT milk (1:50 vol/vol); A, sample collected at the end of the withdrawal period; B, sample collected after 2 days of the end of the withdrawal period; CTRL, control sample; pH_{min}, pH at the end of fermentation process; T_m, time to reach V_m; V_m, maximum acidification rate; ΔpH, $pH_{zero\ time} - pH_{at\ time}$ after 6 and 20 h of incubation.

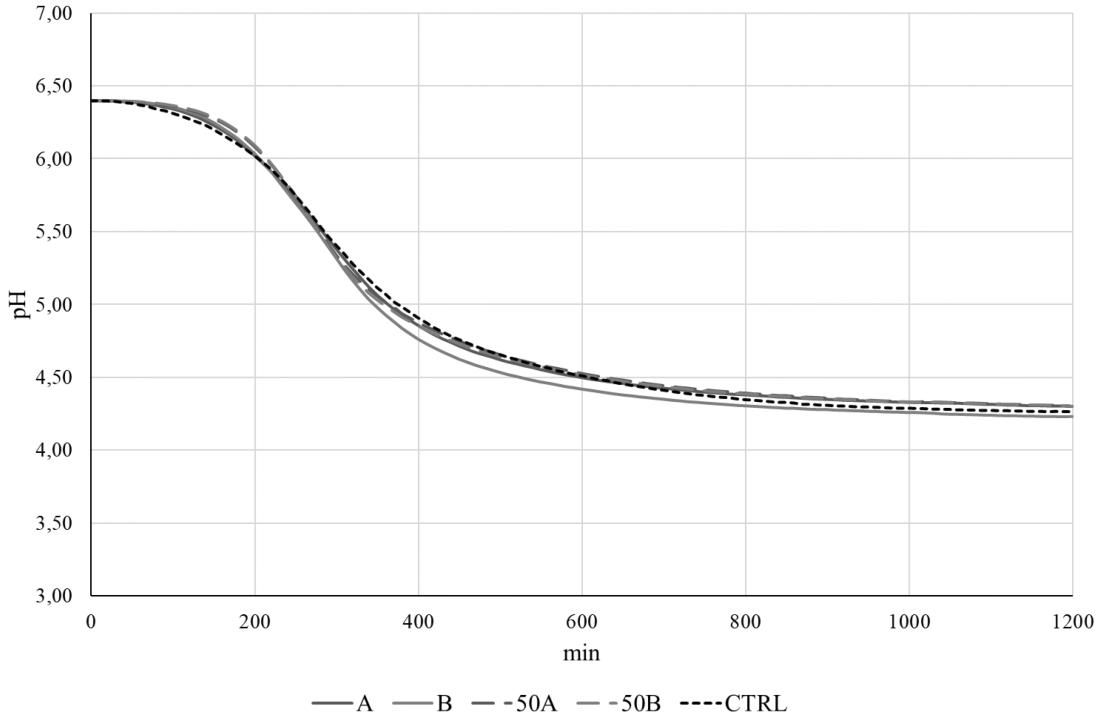

Figure 2 Acidification curves of *Streptococcus thermophilus* ST47 inoculated in: UHT milk (CTRL, control sample), milk collected at the end of the withdrawal period (A), milk collected 2 days after the end of the withdrawal period (B) and in A and B milk diluted 1:50 (vol/vol) in UHT milk (50A, 50B). The average curves are reported.

observed, the impact of each antibiotic on the LAB growth was evaluated.

As shown in Table 4, the use of different antibiotics did not affect the *St. thermophilus* ST47 development. This strain grew rapidly and increased more than 1 log (from 1.2 to 1.5 log) during the first 6 h of incubation. The *St. thermophilus* pH curves appear quite similar (Figure 2), and no significant differences were observed in any of the kinetic parameters of acidification considered herein ($P > 0.05$). Moreover, the pH at the end of the fermentation process achieved comparable values (about pH 4.4) in all conditions tested (Table 4).

Analogues results were obtained when milk samples were inoculated with *Lb. helveticus* DSM 20075 strain. As reported in Table 4, the content and the acidifying capability of *Lb. helveticus* strain did not significantly change in milk samples considered (Figure 3). Comparing the kinetics parameters of acidification, as expected, *Lb. helveticus* reduced milk pH more slowly than *St. thermophilus* (ΔpH at 6 h: 0.4 vs 1.4) but achieved lower pH values at the end of the fermentation process (Table 4). In the experimental conditions tested in this work, the use of antibiotics for cows' treatment did not influence the *Lb. helveticus* DSM 20075 development.

A different scenario was observed with *Lb. delbrueckii* subsp. *bulgaricus* LB08. Even though no significant differences were found in *Lb. delbrueckii* counts during the 20 h of incubation (Table 4), an acidification delay was observed in milk samples deriving from bovines treated with antibiotics. As shown in Figure 3, the fermentation process was more rapid in CTRL samples than in the other ones. The delays in the pH reduction were evident considering the T_m and pH_{min} values that resulted significantly higher in A, B, 50A-B samples (T_m: from 11.1 ± 5.0 to 12.7 ± 5.5 h; pH_{min}: from 4.6 ± 0.7 to 4.8 ± 0.7) than in CTRLs (T_m: 6.9 ± 4.3 h; pH_{min}: 3.9 ± 0.0; Table 4). It is interesting to notice that the dilution of milk samples (1:50 vol/vol), even not significant, caused a slight tendency to speed up the pH fall; in fact, in 50A-B samples, the V_m achievement (T_m) happened 1 h earlier as compared to the no-diluted ones (A and B) (T_m: 11.5 vs 12.7 h) (Table 4).

Differently from those observed in *St. thermophilus* and *Lb. helveticus*, the acidifying capability of *Lb. delbrueckii* subsp. *bulgaricus* seems to be influenced by the cow antibiotic therapy. As shown in Figure 4, the activity of the starter culture in the milk collected from the treated cows was dramatically delayed.

Considering the time required to reduce the pH value by one unit ($T_{\Delta pH}$), the values of this parameter in the control samples (6.5 h) were significantly different from that of the experimental ones ($12.0 < T_{\Delta pH} < 15.5$ h) ($P < 0.05$;

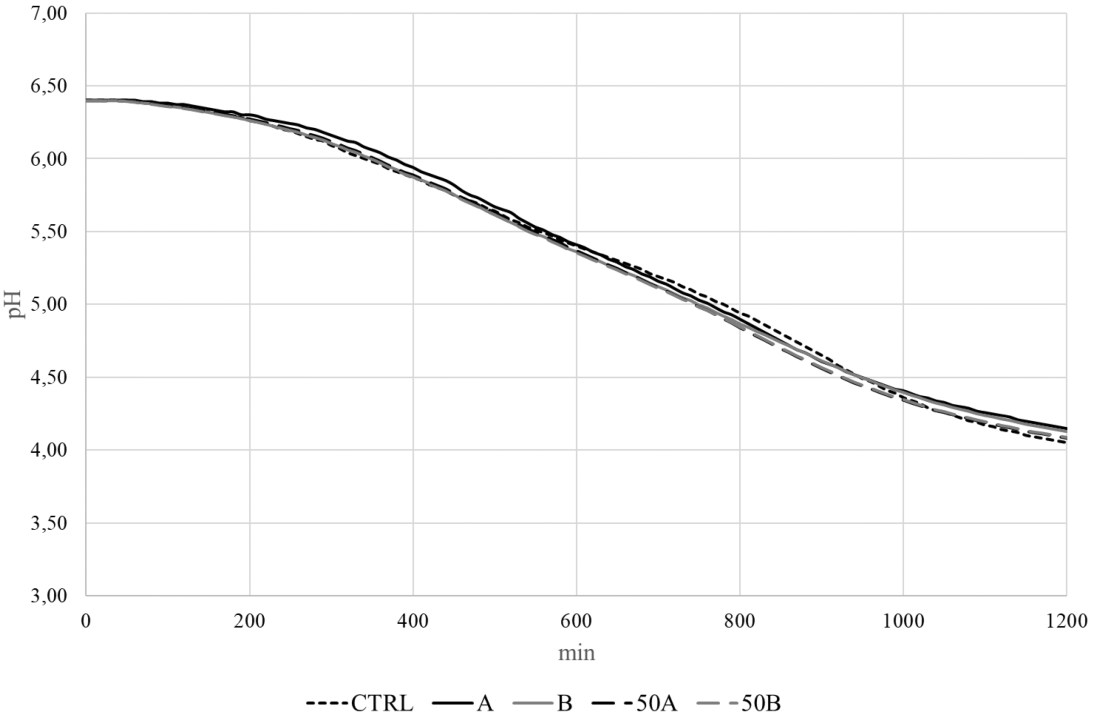

Figure 3 Acidification curves of *Lactobacillus helveticus* DSM 20075 inoculated in: UHT milk (CTRL, control sample), milk collected at the end of the withdrawal period (A), milk collected 2 days after the end of the withdrawal period (B) and in A and B milk diluted 1:50 (vol/vol) in UHT milk (50A, 50B). The average curves are reported.

Figure 5). In particular, sulfonamides and penicillins + clavulanic acid class slowed fermentation by more than 8 h ($T_{\Delta pH}$: 15.5 and 14.3 h), while delays on the order of 5.5 and 6.5 h were observed with penicillins ($T_{\Delta pH}$: 12 h) and cephalosporins ($T_{\Delta pH}$: 13 h).

This suggests that the activity of the starter culture can be affected by the presence of drug residues also after the withdrawal period. In the two antibiotic classes, no significative differences were detected extending the suspension period by 48 h even in samples diluted 1:50 (samples A, B, 50A and 50B).

DISCUSSION

The presence of antibiotic residues in raw milk can be a significant problem since it affects both the dairy technological aspects and consumer safety (Li *et al*. 2019; Virto *et al*. 2022; Climova *et al*. 2024). A recent EU report showed that 0.12% of 9.555 milk samples were positive (above the MRL concentration) for the presence of one or more drugs belonging to the β-lactams, tetracyclines, macrolides, aminoglycosides, sulfonamides and quinolones (EFSA 2019). Positive samples (11) were collected from seven different countries and the percentage of non-compliant milk was in line with those of the previous 11 years.

In the last decade, many researchers studied the impact of different antibiotics used in veterinary field on the acidification activity of the dairy starters. These studies were performed by inoculating LAB cultures in milk spiked with different antibiotic concentrations according to EU MRL (Berruga *et al*. 2008; Chiesa *et al*. 2020; Navrátilova *et al*. 2022; Beltrán *et al*. 2023). So far, few studies have addressed on the effect of antibiotic residues on LAB development in real milk samples. The aim of this study was to evaluate the growth and the acidifying activity of starter cultures in real milk samples collected from cows treated with different classes of antibiotics (β-lactams and sulfonamides) at the end and 2 days after the withdrawal period. Among 45 individual cow milk samples tested by 4Sensor BSTQ assay, no antibiotic residues were detected. These findings agreed with Chiesa *et al*. (2020) that did not detect any amoxicillin, ampicillin, cefalonium, ceftiofur, dicloxacillin, sulfadiazine and trimethoprim residues in 254 Italian cow milk samples. Considering the different milk typologies and their respective controls, no delay in *St. thermophilus* growth and acidification activity was observed. Many studies indicated that *St. thermophilus* starter strains can be resistant to penicillins and sulfonamides (especially trimethoprim) (Zarzecka *et al*. 2020; Nunziata *et al*. 2022). Given that the ampicillin MIC value (23 μg/L) of the strain used

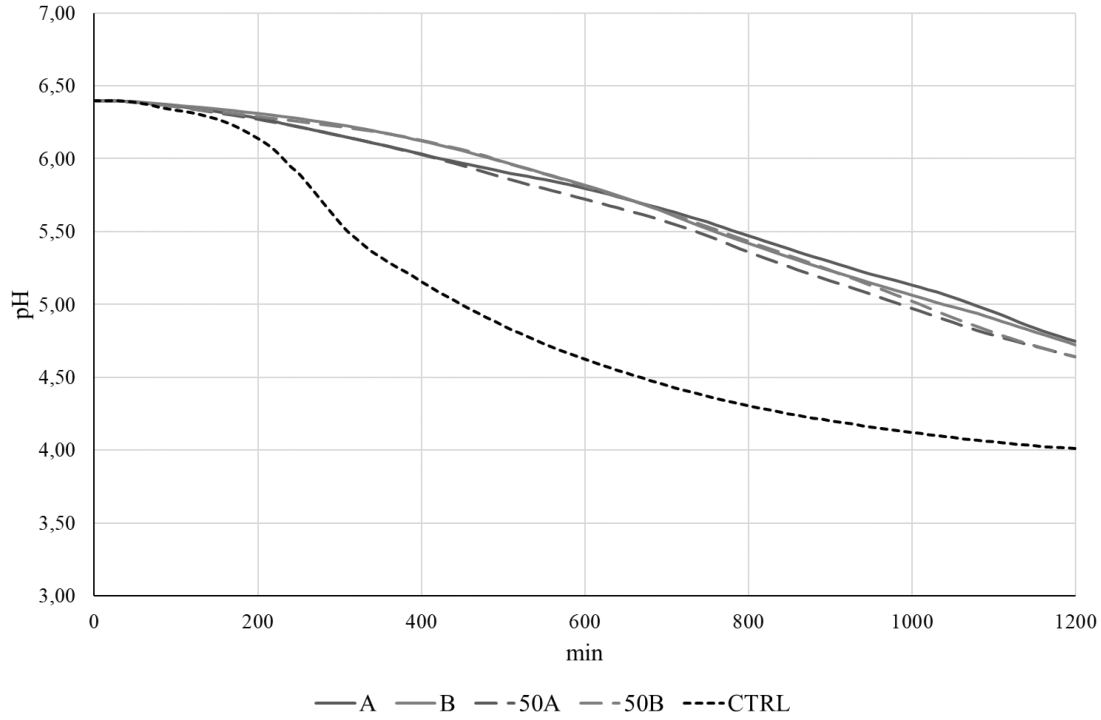

Figure 4 Acidification curves of *Lactobacillus delbrueckii* subsp. *bulgaricus* LB08 inoculated in: UHT milk (CTRL, control sample), milk collected at the end of the withdrawal period (A), milk collected 2 days after the end of the withdrawal period (B) and in A and B milk diluted 1:50 (vol/vol) in UHT milk (50A, 50B). The average curves are reported.

in this study was greater than the MLR limit (4 μg/L), it was able to grow in the milk samples from cows treated with this drug. Moreover, our results are consistent with Berruga *et al*. (2008) who highlighted that real milk samples containing legally admissible amounts of cephalosporins (ceftiofur and cephalexin) did not impact the *St. thermophilus* acidification. On the contrary, the findings of this study contrast with Novés *et al*. (2015) that showed an alteration in the *St. thermophilus* acidification capability in ewe's milk fortified at low concentration (equal to or below the MRL) of cephalosporins (cephalexin). These discrepancies between the *in vitro* (milk artificially spiked with antimicrobials) and *in vivo* (milk collected from animals treated with antibiotics) results could be attributed to the drug pharmacokinetics. Jaglan *et al*. (1992) described that in milk from cows treated with sodium ceftiofur, only 0.1% of the dose of this compound was recovered in milk and less than 35% of its metabolites were microbiologically active, thus underlining its low activity against starter cultures.

Lb. helveticus represents one of the predominant species of the natural whey starter used to produce hard cheeses such as Grana Padano and Parmigiano Reggiano (Morandi *et al*. 2019). As previously described by Giraldo *et al*. (2017) during the cheese-making process, the antibiotic residues present in milk can be transferred to the whey compromising its utilisation as a starter. Our results showed that the *Lb. helveticus* content and its acidifying capability did not significantly change in milk collected at the end of the withdrawal period. These findings could be explained by the tolerance to high concentrations of penicillins and sulfonamides (especially trimethoprim) that characterises the strains belonging to this species (Nunziata *et al*. 2022). As described above for *St. thermophilus, Lb. helveticus* can grow in milk containing legally admissible amounts of ampicillin (4 μg/L), since it showed a MIC value of 125 μg/L. Although cephalosporins are one of the most frequently used antibiotics for dairy cows, few data are available on the resistance of *Lb. helveticus* to these drugs. Recently, Anisimova *et al*. (2022) found that within the *Lb. helveticus* species, the resistance to cephalosporins was highly variable and strain related.

Differently from *St. thermophilus* and *Lb. helveticus*, a significant delay in acidification process was observed in milk samples inoculated with *Lb. delbrueckii* subsp. *bulgaricus*. The decline in acidification activity was not, however, associated with a decrease in viable microbial counts. The kinetic of acidification of this strain was considerably affected in milk samples collected from cows treated with different classes of antibiotics. The delay in the fermentation process was detected also in milk collected 2 days after the

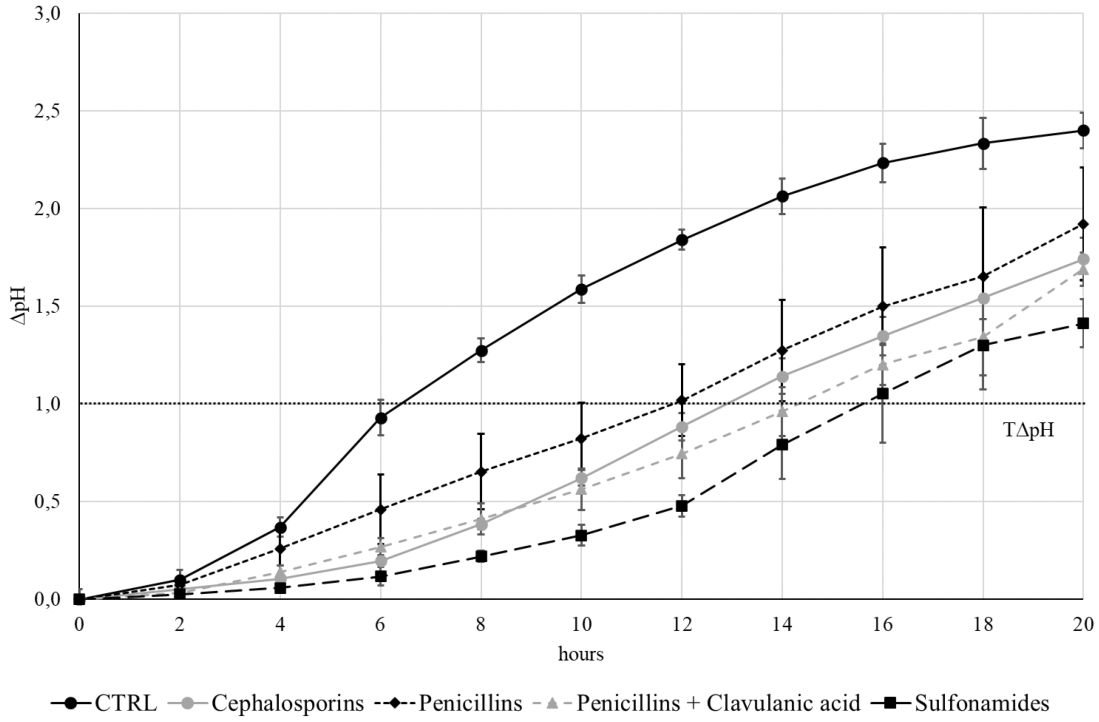

Figure 5 Acidification kinetics of *Lactobacillus delbrueckii* susp. *bulgaricus* in milk from cow treated with different classes of antibiotics. The acidification rate was calculated as ΔpH (ΔpH: pH zero time − pH at time) and was detected every 2 h for 20 h. TΔpH was defined as the time needed to decrease the pH by 1 unit.

withdrawal period and in diluted samples. This suggests that the growth of *Lb. delbrueckii* used in this study was affected also by concentration below the limit of detection of 4Sensor BSTQ assay for cephalosporins (<15 μg/kg), penicillins (<8 μg/kg) and sulfonamides (<10 μg/kg) and their effect persists over time.

Our results are consistent with Navrátilova *et al*. (2022) who observed that the low concentrations of cephalosporins (below the MRL) were able to inhibit the acidifying activity of yogurt cultures, especially, ceftiofur (50 μg/kg) delayed fermentation by 3.5 h compared to the control.

On the other hand, Berruga *et al*. (2008) proved how the ceftiofur significantly may affect the yogurt culture acidification in spiked ewes' milk (50 μg/kg), but in real samples collected at the withdrawal period, no effect on pH reduction was observed during the yogurt fermentation. The findings related to ceftiofur may be of particular importance because this antibiotic is used in dairy cow treatment and is available in formulations with no milk withdrawal period.

Also, clavulanic acid and sulfonamides seem to have a negative effect on the fermentation process, increasing the TΔpH (7.8 and 9 h respectively) as compared to CTRL samples. Although the antibiotic resistances of starter cultures are extensively evaluated, few information is available about these compounds (Nunziata *et al*. 2022). Georgiev *et al*. (2019) highlighted that *Lb. delbrueckii* subsp. *bulgaricus* strains are susceptible to amoxicillin + clavulanic acid while Guo *et al*. (2019) showed that the trimethoprim resistance was strain dependent. Moreover, the impact of antibiotic residues on the growth *Lb. delbrueckii* subsp. *bulgaricus* confirms the findings of recent studies (Morandi *et al*. 2019; Mancini *et al*. 2021), which indicate a drastic reduction or absence of *Lb. delbrueckii* in Grana Padano whey cultures. Our findings suggested that the acidification capability of starter culture can be affected by the use of drugs also after the withdrawal period and could influence the cheese-making process. These compounds can interfere with the development of the milk microbiota, influencing the biodiversity of dairy products and the complex biochemical processes necessary to achieve the final characteristics of the finished cheese. In particular, antibiotic residues can affect the biological processes responsible for the formation of volatile compounds, potentially leading to alterations in the characteristic cheese flavour expected by consumers (Quintanilla *et al*. 2019). Moreover, in the case of raw milk cheeses, an insufficient pH decrease in milk could involve a potential development of spoilage/pathogen bacteria, thus resulting in safety problems, cheese defects and economic losses (Santamarina-García *et al*. 2024).

CONCLUSION

The herein results highlighted that the growth and the acidification capability of starter culture can be affected by the use of drugs, even after the withdrawal period. Moreover, our findings provided evidence that milk from dairy cows treated with antibiotics can adversely affect the development of specific bacteria and pose an important issue of public health, despite the withdrawal period and MRL limits. Further research is needed to verify the potential impact of intake of milk from treated animals on the consumer's gut microbiota.

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AUTHOR CONTRIBUTIONS

Stefano Morandi: Formal analysis; investigation; validation; writing – original draft. **Tiziana Silvetti:** Writing – review and editing; investigation. **Matteo Guerci:** Conceptualization; supervision. **Alberto Tamburini:** Writing – review and editing; software. **Milena Brasca:** Conceptualization; funding acquisition; writing – review and editing; project administration.

CONFLICT OF INTEREST

The authors state that there is no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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