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Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process

Luca Maria Chiesa [a], Lucia DeCastelli [b], Maria Nobile [a,*], Francesca Martucci [b], Giacomo Mosconi [a], Mauro Fontana [c], Marta Castrica [a], Francesco Arioli [a], Sara Panseri [a]

[a] Department of Health, Animal Science and Food Safety, University of Milan, Via Celoria 10, 20133, Milan, Italy
[b] Istituto Zooprofilattico Sperimentale Piemonte, Liguria e Valle d'Aosta, Via Bologna, 148, 10154, Turin, Italy
[c] DVM, Specialist in Inspection of Food of Animal Origin, Turin, Italy

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ABSTRACT

Antibiotics are widely used in livestock production as disease treatment, prevention and improve feeding efficiency. In this study three screenings and a multiclass method by Liquid Chromatography tandem Mass High Resolution Spectrometry (LC-HRMS) were developed and used to evaluate the antimicrobial substance incidence in 254 raw bovine milks involved in PDO cheese production in Northern Italy. The LC-HRMS results were compared to assess the frequency of false and negative results in term of reliability of screening methods. An investigation in relation to the quantified residues evaluating their possible negative impact on milk starter cultures in a simulated cheese-making process was presented. Lincomycin residues were observed in 30 samples, with 11.8% frequency and 17.29 ppb as mean value below its MRL. Three samples showed oxytetracycline respectively at 15.05, 0.82 and 1.59 ppb and two cefapirin and spyramycin at trace level. False positives with an acceptable frequency were observed by using the 3 kits confirming this approach useful for monitoring plans. Considering lincomycin, negative effect was demonstrated toward lactic acid bacteria activity in term of bacteria counts, pH and acidity during cheese making simulation. This represents a critical aspect considering both the economic value of PDO cheeses and the antibiotic resistance diffusion.

1. Introduction

Antibiotics are widely used in livestock production for different purposes such as disease treatment, prevention and to improve feeding efficiency (growing promoters) (Rama, Lucatello, Benetti, Galina, & Bajraktari, 2017). As reported by Bacanlı and Başaran (2019), the 80% of the animals involved in food production are currently being treated with veterinary drugs at a certain time or throughout their lives (Bacanlı & Başaran, 2019). As direct consequence, antibiotic usage in animals may leave antibiotic residues in foodstuffs such as milk, egg and meat. Milk consumption is large-scale throughout the world and was estimated at approximately 900 million tons in the year 2018 (Dairy, Goetz, Diepenbrock, & Wyrzykowski, 2019, pp. 1–4). As a consequence, milk safety represents a critical issue. In general, chemicals are considered the main contaminant since they are directly introduced during dairy management and the milking process (Faustini et al., 2019). Tetracycline, β-lactams, quinolones, sulfonamides, streptomycin and chloramphenicol are the most frequently used antibiotics in dairy cattle and their residues in milk would adversely impact human health by increasing the risk of allergies in the susceptible population and the development of resistant bacteria (Jank et al., 2015; Darwish et al., 2013). For human health safety, maximum residue limits (MRLs), typically ranging from 4 to 1500 μg kg^{-1}, are set for different classes of antibiotics as the residue can remain in animal-origin food (Gaudin, 2017). Residue levels within MRLs have no adverse health effects if ingested daily by humans throughout their lifetime but the use of antimicrobials in livestock production and their role in the development of antimicrobial resistance represent a public health concern (Ammar, El-Shazly, Zalma, & El-Sharoud, 2018). The most known antibiotic resistant strains are antibiotic resistant salmonellae, macrolide or fluoroquinolone resistant campylobacters, glycopeptide or streptogramin resistant enterococci and multiple antibiotic resistant Escherichia coli (Tempini, Aly, Karle, & Pereira, 2018). The European Food Safety Authority (EFSA) collects and examines the data in official reports on antibiotic resistance from EU Member. Cephalosporins, macrolides, polymyxins and quinolones are in the list of critically important antibiotics, suggesting that their incidence

* Corresponding author.
E-mail address: maria.nobile1@unimi.it (M. Nobile).

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in milk be monitored (Food & Authority, 2016). In addition to antibiotic resistance the technological impact in the dairy sector must also be considered since antimicrobial drugs can interfere with the production of dairy products, decreasing acid formation, reducing the curdling of milk and causing an improper ripening of cheeses (Quintanilla, Paloma, Beltrán, Peris, Rodríguez, & Molina, 2018). This is crucial for some geographical areas where milk production is primarily destined for PDO or IGP cheese-making (Parmigiano Reggiano and Grana Padano) even if

Table 1
Literature survey on analytical methods developed to determine different antimicrobial agent residues in milk.

Reference	Compounds Examined	Milk typology/n° of samples	Extraction Technique	Detection technique	LOD/LOQ CCα/CCβ	Min and Max concentration range (Application)
Thompson et al. (2011)	lasalocid, monensin, narasin, and salinomycin	1072 Canadian raw bovine milk	L/L extraction with acetonitrile	LC-MS/MS	LOQ 0.1 ng g^{-1}	0.10–0.53 ng g^{-1} of monensin
Aalipour et al. (2013)	Penicilins Sulfonamids Tetracyclins Streptomycin Lincomycin Erythromycin Tylosin Ceftifours Gentamycin Neomycin	187 pasteurized and sterilized commercial Iranian milk	Incubation	Microbiological detection test kit	LOD 2–200 ng g^{-1}	Prevalence % of antibiotic contamination based on season variation and thermal processing methods
Jank et al. (2015)	5 macrolides and 2 lincosamides	Brazilian bovine milk	L/L extraction with acetonitrile	LC–MS/MS	LOD 5–25 ng mL^{-1} LOQ 10–37.5 ng mL^{-1}	No application
Pogurschi, Ciric, Zugrav, and Patrascu (2015)	4 tetracyclines, 3 beta-lactams	210 raw milk of Bucharest	pH adjustment and incubation	Beta Star Combo immuno test	LOD 2–75 ng g^{-1}	11% beta-lactam, 82% tetracycline positives
Bion et al. (2015)	7 penicillins, 7cephalosporins, 3 tetracyclines, 6 sulfonamides, 2 Aminoglycoside, 1 macrolide	cow's milk, skimmed and full cream milk powders	incubation	Delvotest® T	screening target concentration 2–100 ng g^{-1}	No application
Rama et al. (2017)	β-lactams, tetracycline, and sulfonamides for the screening test, 5 β-lactams for confirmation	1734 raw milk of Kosovo	Extraction with acetonitrile, defatting, STRATA X-SPE	Delvotest SP assay and an enzyme-linked receptor-binding assay (SNAP) and confirmation by LC-MS/MS	CCα 4.9–35.8 ng g^{-1} CCβ 5.5–41.6 ng g^{-1}	2.1–1973 ng $g^{-1}$of penicillins
Giraldo et al. (2017)	8 β –lactams, 2 tetracyclines, 2 sulfonamides, 2 quinolones, 2 aminoglycosides, 2 macrolides	Raw goat milk and cheese samples	/	Eclipse 100 microbial inhibitor test	CCβ 2.4–826.9 ng g^{-1}	Evaluation of antimicrobial activity of the whey
Zhou et al. (2017)	16 macrolide antibiotics and 4 metabolites	60 commercial Chinese milk samples	QuEChERS	LC–MS/MS	LOD 0.30–0.85 ng g^{-1} LOQ 1.1–4.0 ng g^{-1}	8.38–28.18 ng g^{-1}
Li et al. (2018)	8 β-lactams, 5 quinolones, 3 phenicols, and 1 nitrofuran	20 commercial milk samples	Extraction with acetonitrile, defatting	LC–MS/MS	CCα 0.008–113.68 ng g^{-1} CCβ 0.01–125.65 ng g^{-1}	Not detected
László et al. (2018)	3 penicillins, 2 sulfonamides, 2 aminoglycosides, 2 cephalosporins and tetracycline	Raw milk	L/L extraction with acetonitrile	LC–MS/MS	LOD 0.02–58 ng mL^{-1} LOQ 0.5–200 ng mL^{-1}	study of heat degradation kinetics
Tempini et al. (2018)	5 β-lactams, 4 tetracyclines, 3 quinolones, 8 sulfonamides, 1 benzimidazole, 3 macrolides	25 waste milk from dairy farms of Central California	Extraction with acetonitrile and Oasis HLB SPE	SNAP Beta-Lactam ST test, LC–MS/MS	LOQ 2–250 ng mL^{-1}	7–590 ng $mL^{-1}$of β-lactams, sulfonamides, tetracyclines. Evaluation also of milk quality parameters and antimicrobial susceptibility
Quintanilla, Beltrán, Peris, Rodríguez, and Molina (2018)	3 macrolides	Goat's milk and cheese	Not specified	LC–MS/MS	LOQ 10 ng g^{-1}	198.7–1539.8 ng g^{-1}
Delatour et al. (2018)	3 amphenicols, 14 benzimidazoles, 11 coccidiostats, 2 diaminopyrimidines, 2 lincosamides, 8 macrolides, 17 quinolones, 21 sulfonamides, 2 rifamycins	1 fat-filled milk, 33 Milk-based products, 4 milk powders, 14 infant formulae	Extraction with acetonitrile, formic Acid and Na_2SO_4, purification with Na_2SO_4/PSA/C18	Screening strategy based on an analyte-specific correction of the matrix effect (SACME), LC-MS/MS	Screening target concentration 0.2–15 ng g^{-1}	No application
Han et al. (2019)	14 sulfonamides, 13 β-lactams, 10 quinolones, 4 tetracyclines, 1 aminoglycoside, 1 amphenicol	milk	pH adjustment and incubation	lateral flow immunoassay	LOD 0.04–1.1 ng mL^{-1}	No application
Wu et al. (2019)	13 β-lactams, 6 aminoglycosides, 4 tetracyclines, 6 sulfonamides, 4 macrolides, 1 lincosamide	100 Goat milk	Inoculation and incubation	Microbiological Inhibition Test	LOD 2–1250 ng mL^{-1}	Validation and comparison of this microbiological system to other commercially available microbiological methods

scarce literature is currently available (Pretto, Marchi, De, Penasa, & Cassandro, 2013). In this context a residue concentration even below the MRL can lead to a negative impact on cheese, causing economic losses (Pogurschi Ciric, Zugrav, & Patrascu, 2015). Some researchers have also investigated the role of temperature treatment as possible residue inactivation even if this matter is controversial (László, Lányi, & Laczay, 2018; Mirlohi, Aalipour, & Jalali, 2013). Many of these studies have reported that partial degradation of β-lactams, quinolones, sulfonamides, macrolides, tetracyclines and aminoglycosides is temperature-dependent and prolonged heating time helps to induce more degradation (Ianni et al., 2018) Antibiotics in milk could also be retained in milk curd to a greater or lesser extent in relation to their physicochemical properties and ability to interact with fat and/or proteins of milk during cheese-making as demonstrated by Hill, 2000. This phenomenon is important since cheese-making by-products such as whey are currently recycled in foodstuff manufacturing and are also used for animal feeding (Castrica et al., 2019). As regards antibiotic residue determination in milk, another critical point is the need of multiclass robust and sensitive protocols (Martins et al., 2016). The modern approach involves the possible use of screening and confirmatory methods. Screening methods are defined as "methods used to detect the presence of an analyte or class of analytes at the level of interest" (European Parliament and the Council of the European Union, 2002). Their different detection principles include either microbiological or biochemical interactions (Wu et al., 2019). Microbiological tests can be performed by non-professionals, are usually very fast but have some disadvantages such as lack of specificity and long incubation time required. Instead, confirmatory methods typically based on Liquid Chromatography tandem Mass Spectrometry (LC-MS) detection require tedious sample preparation but are generally characterised by high sensitivity (Delatour et al., 2018; Tsagkaris et al., 2019). Many extraction procedures for milk have been described, such as protein precipitation and hot water as extraction solvent, solid phase extraction (SPE), and QuEChERS (Shendy, Al-Ghobashy, Gad Alla, & Lotfy, 2016). An overview of recent literature highlighting the analytical methods used to investigate the presences of antibiotic residues in milk is presented in Table 1. However, most published methods have strong limitations since they are not able to detect multiclass residues, essential for setting up monitoring plans (Zhang et al., 2019). Therefore, it is highly important to develop fast and economical confirmation methods to simultaneously multiclass residues in milk using a single procedure (Jank et al., 2015). In view of the above-mentioned considerations the aims of this research were:

- to develop an LC-HRMS method based on multiclass antibiotic detection in raw bovine milk used for PDO cheese production in Northern Italy to assess and evaluate the incidence of antimicrobial substances in relation to food safety criteria also comparing the results with those obtained by screening methods,
- to investigate their impact on milk starter cultures, based on quantified residues, even if detected below their respective MRLs, in a simulated cheese-making process of a PDO cheese. This aspect is critical considering both the economic value of PDO cheeses and the persistence of antibiotic residues which contribute to antibiotic resistance diffusion.

2. Materials and methods

2.1. Chemicals and reagents

The solvents and reagents were obtained from Merck (KGaA, Darmstadt, Germany). All antimicrobial agents investigated (Table 2) and the internal standard (IS) were purchased from Merck. The Solid Phase extraction (SPE) cartridges (Oasis HLB 3 mL, 60 mg), were provided by Waters (Milford, Massachusetts, United States). Kits for screening method were purchased from the market: Delvotest® SP NT plates from DSM (Heerlen, the Netherlands), ROSA Charm QUAD1 Test from Charm Sciences Inc (Lawrence, Massachusetts, United States) and Milk Antibiotic Testing 3 in 1 Macrolides Erythromycin – Lincomycin – Tylosin – Tilmicosin 96 Tests from Shenzhen Bioeasy Biotechnology Co., Ltd. (Shenzhen, China). Stock solutions of all antimicrobial agents were prepared in methanol at concentration of 1 mg mL^{-1} and working solutions at 10 and 100 ng mL^{-1}, kept at −20 °C.

2.2. Milk samples

254 raw bovine samples were collected during the year 2019 (January–November) and processed simultaneously by using three screening methods and confirmation analyses based on mass-spectrometry detection to investigate the frequencies and concentrations of possible antimicrobial residues in order to assess their conformity with food safety criteria. Then, based on the residues quantified in milk samples, a cheese making simulation by using milk fortified with different antibiotic concentration to assess the impact of residues on milk production. The entire experimental plan with working phases was presented in Fig. 1.All samples were obtained from local farms located in North Italy (Piedmont Region), a geographical area in which the majority of milk is used for Grana Padano PDO cheese production. Grana Padano PDO cheese together with Parmigiano Reggiano PDO is a hard cheese produced in Italy, exported around the world (Moio & Addeo, 1998). Only raw partially skimmed milk from the Grana Padano production area can be used. After natural separation of the cream, the milk is poured into traditional copper vats and processed: a natural whey starter, deriving from the previous day's cheese-making, is added along with pure calf rennet. Once coagulation has occurred, the curd is chopped into small grains with the aid of a manual instrument called "spino". Heating to 53 °C and then, after a resting period of around an hour, the twin fresh wheels of cheese are collected, wrapped in linen cloths and placed into moulds, where they receive the initial mark of origin: small lozenges with alternatively "Grana" and "Padano" written inside and all the other signs appearing on the crust except for the firebrand. Finally, before the ageing process begins, the wheels are soaked in brine for around 23 days. The ageing process lasts from a minimum of 9 to over 24 months. At 9 months, each wheel is carefully tested for appearance, aroma and texture. This step is carried out exclusively by the impartial expertise of the Consorzio Tutela Grana Padano (Protection Consortium) technicians. This Protection Consortium, founded in 1954 and including all producers, is not only responsible for the quality of each wheel, but also promotes and protects the name Grana Padano around the world.

2.3. Antimicrobial residue analysis

Considering the research aims, three combined screening methods each dedicated to the detection of different compounds or antibiotic classes were first used to test the raw bovine milk samples and then compared to the confirmatory multiclass method based on Liquid –chromatography coupled to High Resolution Mass Spectrometry (LC-HRMS) to detect 66 antimicrobial agents. This choice was adopted to confirm the results obtained by the screening tests and to define the feasibility of using rapid screening tests to set up a preliminary monitoring plan in milk sector.

2.3.1. Screening methods analyses

Three different test kits were used following the instructions of supplier. Briefly, Delvotest® SP NT is constituted by 96 wells plates with *Bacillus stearothermophilus var. calidolactis;* 100 μl of milk sample, positive and negative controls were dispensed in each well; plates were incubated, lidded water bath preheated to 64 °C for 3 h and the results were evaluated immediately after incubation. Charm QUAD1 Test is a rapid one step immunoreceptor assay using lateral flow technology. 300 μl of milk sample was deposed into the sample compartment, the strip

Table 2
Validation results with reference to legislation on their MRLs for LC-HRMS method used for antibiotic detection in milk samples.

Chemical Class	Compound	CCα (ng mL^{-1}) Decision limit	CCβ (ng mL^{-1}) Detection capability	Recovery %	CV intra-day %	CV inter-day %	REG. 37/2010/CE MRLs (μg kg^{-1})
QUINOLON	Enrofloxacin	0.10	0.23	98	9	11	100
QUINOLON	Ciprofloxacin	0.11	0.25	96	10	13	100
QUINOLON	Difloxacin	0.11	0.24	93	12	16	Not for use in animals from which milk is produced for human consumption
QUINOLON	Danofloxacin	0.13	0.27	95	11	13	30
QUINOLON	Levofloxacin	0.12	0.22	94	10	12	No MRL
QUINOLON	Lomefloxacin	0.11	0.2	93	11	13	No MRL
QUINOLON	Marbofloxacin	0.13	0.25	96	10	15	75
QUINOLON	Norfloxacin	0.12	0.24	95	13	18	No MRL
QUINOLON	Enoxacin	0.13	0.26	94	12	18	No MRL
QUINOLON	Flumequine	0.11	0.24	97	12	20	50
QUINOLON	Nadifloxacin	0.11	0.25	95	14	17	No MRL
QUINOLON	Oxolinic acid	0.22	0.31	96	12	15	Not for use in animals from which milk is produced for human consumption
QUINOLON	Nalidixicacid	0.24	0.33	94	11	15	No MRL
β-LACTAM	Amoxicillin	0.50	0.62	89	14	17	4
β-LACTAM	Ampicillin	0.53	0.63	90	14	18	4
β-LACTAM	Phenoxymethylpenicillin	0.54	0.64	93	13	16	No LMR
β-LACTAM	Benzylpenicillin	0.54	0.66	94	12	16	4
β-LACTAM	Cefadroxil	4.37	5.10	89	11	16	No MRL
β-LACTAM	Cefalexin	0.49	0.60	92	14	20	100
β-LACTAM	Cefalonium	4.28	5.08	91	14	20	20
β-LACTAM	Cefalothin	4.25	5.06	91	13	18	No MRL
β-LACTAM	Cefazolin	4.30	5.11	88	12	15	50
β-LACTAM	Cefoperazone	4.12	4.99	92	13	15	50
β-LACTAM	Cefquinome	0.47	0.59	93	10	14	20
β-LACTAM	Cefapirin	4.06	5.09	91	11	13	60
β-LACTAM	Ceftiofur	0.18	0.30	93	11	15	100
β-LACTAM	Desfuroylceftiofur	0.21	0.31	94	14	19	100
β-LACTAM	Cloxacillin	0.57	0.72	92	13	19	30
β-LACTAM	Dicloxacillin	0.59	0.75	92	14	17	30
β-LACTAM	Desacetylcefapirin	4.01	5.04	94	12	18	60
β-LACTAM	Nafcillin	0.10	0.22	96	12	15	30
β-LACTAM	Oxacillin	0.11	0.24	89	11	14	30
β-LACTAM	Piperacillin	0.11	0.23	95	10	12	No MRL
MACROLIDE	Tylosin	0.13	0.25	93	10	14	50
MACROLIDE	Tilmicosin	0.12	0.25	94	8	12	50
MACROLIDE	Oleandomycin	0.89	1.04	92	11	16	No MRL
MACROLIDE	Spiramycin	0.39	0.50	87	14	17	200
MACROLIDE	Neospiramycin	0.85	1.02	91	13	17	20
MACROLIDE	Kitasamycin	0.90	1.04	93	12	15	No MRL
MACROLIDE	Josamycin	0.87	1.05	96	12	16	No MRL
MACROLIDE	Tulathromycin	0.38	0.51	95	12	18	No MRL
MACROLIDE	Erythromicyn A	0.40	0.49	95	11	14	40
SULFONAMIDE	Sulfadiazine	0.10	0.22	97	8	13	100
SULFONAMIDE	Sulfadimethoxine	0.12	0.23	98	9	12	100
SULFONAMIDE	Sulfadimidine	0.12	0.25	97	9	11	100
SULFONAMIDE	Sulfamerazine	0.11	0.24	97	10	12	100
SULFONAMIDE	Sulfamethoxazole	0.11	0.24	96	11	13	100
SULFONAMIDE	Sulfamonomethoxine	0.11	0.22	98	10	13	100
SULFONAMIDE	Sulfapirydine	0.13	0.25	98	9	11	100
SULFONAMIDE	Sulfatiazole	0.13	0.24	99	9	12	100
SULFONAMIDE	Trimethoprim	0.10	0.22	99	10	13	100
TETRACYCLINE	Chlorotetracycline	0.15	0.24	96	10	12	100
TETRACYCLINE	Oxytetracycline	0.10	0.22	97	10	13	100
TETRACYCLINE	Tetracycline	0.12	0.24	95	12	15	100
TETRACYCLINE	Doxyicline	0.12	0.23	96	11	14	Not for use in animals from which milk is produced for human consumption
LYNCOSAMIDE	Lincomycin	0.09	0.20	98	7	11	150
AMPHENICOL	Chloramphenicol	0.06	0.12	91	13	14	Prohibited
AMPHENICOL	Tiamphenicol	0.11	0.26	93	11	14	50
AMPHENICOL	Florfenicol	0.21	0.3	92	14	18	Not for use in animals from which milk is produced for human consumption
AMPHENICOL	Florfenicol amine	0.20	0.30	90	13	20	Not for use in animals from which milk is produced for human consumption
PLEUROMUTILIN	Tiamulin	0.18	0.25	94	13	20	No MRL
PLEUROMUTILIN	Valnemulin	0.88	1.02	93	12	16	No MRL
NITROIMIDAZOLE	Dimetridazole	0.10	0.22	93	12	14	Prohibited
NITROIMIDAZOLE	Ronidazole	0.10	0.21	94	14	15	Prohibited
NITROIMIDAZOLE	Tinidazole	0.12	0.24	96	11	16	No MRL
NITROIMIDAZOLE	Metronidazole	0.10	0.21	95	12	15	Prohibited

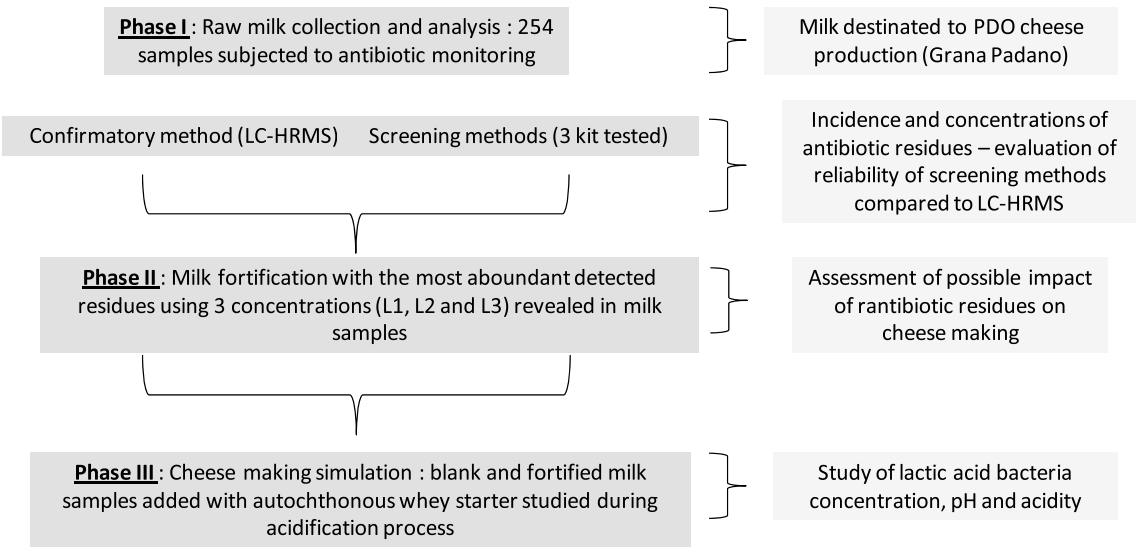

Fig. 1. Experimental phases of the present research.

was incubated at 56 °C for 5 min and the result was evaluated immediately after incubation. Bioeasy Milk Antibiotic Testing 3in1 (Erytromycin+Lyncomycin+Tylosin & Tilmicosin) is a rapid one step immunoreceptor assay based on lateral flow technology. 200 μl of milk sample was deposed into the microwell and incubated at 40 °C for 3 min; a dipstick was inserted in the microwell for 7 min at 40 °C and the result was evaluated immediately after incubation.

2.3.2. LC-HRMS analyses for the detection of antibiotic residues

Milk sample extraction and purification were conducted in duplicate following the procedure described in our previous work (Chiesa, Nobile, Panseri, & Arioli, 2018a). Briefly, 1 ml of raw milk was spiked with the IS at 2 ng g^{-1}, extracted with 5 mL of McIlvaine buffer (pH 4.0) and 100 μL, 20% w/v of Trichloroacetic acid for protein precipitation. After vortex, sonication for 10 min and centrifugation (2500×g, 4 °C, 10 min), the supernatant was defatted with 2 × 3 mL of n-hexane. After SPE preconditioning with 3 mL of methanol and 3 mL of Milli-Q water, the supernatant was loaded and then washed with 2 × 3 mL methanol:water (5:95 v/v); finally 5 mL of methanol were added to elute. The eluate was evaporated and reconstituted in 200 μL of methanol: 0,1%formic acid (10:90 v/v). The analyses were performed by an HPLC system (Thermo Fisher Scientific, San Jose, CA, USA) coupled to a Thermo Q-Exactive Orbitrap (Thermo Fisher Scientific). All the MS parameters, regarding the full-scan (FS) acquisition, combined with the data-independent acquisition (DIA), for the confirmatory response were described in previous work (Chiesa, Panseri, Nobile, Ceriani, & Arioli, 2018b).

2.3.3. LC-HRMS method validation

Method validation was assessed according to the Commission Decision 2002/657/EC guidelines (European Union, 2002) and SANCO/2004/2726 revision 4 (European commission, 2004) as also described in Chiesa et al. (2018b). In particular, the recovery was calculated as the percentage of the true concentration of a substance recovered during the analytical procedure; the decision limit (CCα) and detection capability (CCβ) were calculated to assess our method sensitivity; the precision, in terms of intra- and inter-day repeatability, was evaluated by calculating the coefficient of variation (CV%) obtained for six replicates of each analyte during the single batch of the daily validation and among the different validation series performed in three different days.

2.4. Antimicrobial trials in simulated cheese making

Three concentrations of lincomycin were involved in a laboratory-scale process to simulate the original cheese-making process with a particular focus on the first phase (milk acidification and subsequent addition of whey starter) in order to assess the impact of antibiotic residues. Antimicrobial agents were selected on the basis of major revealed and quantified residues obtained from the current monitoring plan. Three lincomicyn concentrations in triplicate were selected to fortify blank milk: blank (B), L1 (fortified with 10 ppb), L2 (fortified with 20 ppb) and L3 (fortified with 40 ppb). Blank milk was selected from samples previously analysed based on its absence of any antibiotic residues. To investigate the impact of lincomycin on milk cheese-making, 500 mL of milk respectively of B, L1, L2 and L3 were used; 1% (weight/weight) of natural whey starter was added to every sample to simulate technological cheese production parameters, and these samples were placed in thermostatic incubation at 45 °C for 14 h. The whey is a natural one, obtained from the processing with semi-fat hard cheese of the previous day; the whey microflora is therefore autochthonous and does not derive from the use of starter cultures. The acidification kinetic of the milk obtained at the end of the acidification process prior to receiving rennet was investigated periodically using a pH-meter (model Basic 20, Crison, Barcelona, Spain) with a penetration probe (model 5232, Crison, Barcelona, Spain).

At the end of the acidification process during which whey starter was initially added to the milk, lactic acid bacteria and total microbial count were determined to investigate their possible inactivation or reduction effects to confirm the negative impact of lincomycin on cheese-making process.

2.4.1. pH determination, acidity and microbiological analyses

The microbiology tests and pH determination were used to check lactic acid bacteria and total microbial count useful to investigate possible inactivation or reduction effects on cheese making.

The following microbiological parameters were determined in different samples of milk (10 mL) added with whey starter and fortified with different concentrations of lincomycin (10, 20, 40 ng g^{-1}), after homogenization in 90 mL of sterile diluent solution (0.85% NaCl and 0.1% peptone), and homogenized in a stomacher for 1 min at room temperature and then serial 10-fold dilutions were prepared in a sterile saline solution. Mesophilic aerobic bacteria and presumptive *Lactococcus* and *Lactobacillus* species. In particular, Mesophilic aerobic bacteria were determined using Plate Count Agar (Oxoid CM0325) and then the plates were incubated at 30 °C for 72 h. Lactic streptococci were determined using M17 agar and then the plates were incubated at 37° ± 1 °C for 2 days. Total *Lactobacillus* species were determined using MRS

agar and incubated at 30 °C ± 1 °C for 72 ± 3 h under microaerobic atmosphere. The reading of the plates was made through the use of the tool: Interscience - Scan® 4000 and the result were expressed as colony-forming unit (CFU) mL^{-1}. Moreover, pH trends during 12 h acidification process as well as acidity at the end of phase were determined. Titratable milk acidity was determined according to official methods for milk AOAC, 2006 and expressed as °SH/50 mL. All samples were prepared and analysed in duplicate.

3. Results and discussions

3.1. Analytical methods validation

3.1.1. Screening method validation parameters

Validation parameters (LOD) for the three methods are presented in Table 3. In detail, Delvotest® SP NT test is an AOAC Performance Tested Method (Certificate Nr. 011102) and the laboratory verified internal performance; the Charm QUAD1 Test was validated directly by the laboratory; the Bioeasy Milk Antibiotic Testing 3in1 is currently undergoing validation by the laboratory. According with the guidelines described by the Commission Decision 2002/657/CE concerning the performance of analytical methods and the interpretation of results, for intra-laboratory validation purposes, the following parameters were taken into consideration: specificity, robustness and the CCβ detection limit that was evaluated, when possible, at half of the MRLs, as indicated by the Reg. 37/2010 CE.

Verification of these parameters is necessary before a screening test can be applied in an official analysis laboratory.

3.1.2. LC-HRMS validation parameters

All validation parameters are summarised in Table 2. Good performance of the method was demonstrated by CCα and CCβ much lower than MRLs. Recoveries ranged from 88 to 99% and the CV% were all ≤20% (European Union, 2002). At present, scarce confirmation methods are available for the simultaneous detection of 66 antimicrobial agents belonging to different chemical classes in raw milk as also highlighted in Table 1. Examining the literature available, it can be seen that the other methods reported are of a screening type or for confirmation but by low resolution MS. The peculiarities of the method presented in this study can be highlighted in terms of selectivity and specificity for a very varied multiclass method and are among those with the highest number of antibiotics analysed. Our limits are in most cases among the lowest comparing them with those by LC-MS/MS shown in Table 1. As an example, our macrolide limits ranged from 0.12 to 0.90 ng mL^{-1} if compared with the study of Quintanilla, Beltrán, Molina, Escriche, and Molina (2019), which dealt only with macrolide class analysis, that ranged from 10 to 30 ng g^{-1}, while our macrolides results are comparable with those of Zhou et al., 2017; our tetracyclines ranged from 0.10 to 0.15 ng mL^{-1} if compared with the 10 ng mL^{-1} of Tempini et al. (2018). The effectiveness of our validation data by HRMS showed almost zero background in the extracted ion parent chromatograms and this is very important for complex and heterogeneous matrices rich in interferences, where the matrix effect can be substantial, as discussed in the study by Delatour et al. (2018). Moreover, accurate exact mass had a crucial role in identifying a compound with absolute certainty and in its ability to discriminate from matrix interferences (Fig. 2) which could potentially lead to false positive detections, if analysed with screening techniques or by using low MS resolution instruments. This advantage can also be exploited together with the ability to retrospectively observe the presence of possible metabolites or degradation products that may be found in the samples, enriching multiresidual research with further information. Obviously, sample preparation and purification play an important role in the success of the analysis, allowing excellent instrument performance.

Table 3
Validation results for the three screening tests used for milk analyses.

Method: Delvotest® SP NT			
Chemical Class	Compound	CCβ (ng mL^{-1}) Detection capability	REG. 37/2010/CE MRLs (μg kg^{-1})
β-LACTAM	Amoxicillin	2.5	4
β-LACTAM	Ampicillin	2.5	4
β-LACTAM	Benzylpenicillin	2	4
β-LACTAM	Cefalexin	50	100
β-LACTAM	Cefapirin	5.8	60
β-LACTAM	Cloxacillin	20	30
β-LACTAM	Dicloxacillin	15	30
β-LACTAM	Oxacillin	10	30
MACROLIDE	Tylosin	25	50
MACROLIDE	Spiramycin	400	200
MACROLIDE	Erythromicyn A	200	No MRL
SULFONAMIDE	Sulfadiazine	100	100
SULFONAMIDE	Sulfametazine	150	100
SULFONAMIDE	Trimethoprim	250	100
TETRACYCLINE	Chlorotetracycline	400	100
TETRACYCLINE	Oxytetracycline	200	100
TETRACYCLINE	Tetracycline	200	100
LYNCOSAMIDE	Lincomycin	150	150
Method: Charm Quad 1 ROSA Test			
Chemical Class	**Compound**	**CCβ (ng mL-1) Detection capability**	**REG. 37/2010/CE MRLs (μg kg-1)**
QUINOLON	Enrofloxacin	10–15	100
QUINOLON	Ciprofloxacin	10–15	100
QUINOLON	Danofloxacin	15–20	30
QUINOLON	Oflaxacin	10–15	No MRL
QUINOLON	Lomefloxacin	10–15	No MRL
QUINOLON	Marbofloxacin	20–30	75
QUINOLON	Norfloxacin	5–10	No MRL
QUINOLON	Flumequine	20–40	50
QUINOLON	Pefloxacin	5–10	No MRL
QUINOLON	Orbifloxacin	5–10	No MRL
QUINOLON	Nalidixic acid	10–15	No MRL
β-LACTAM	Amoxicillin	2–4	4
β-LACTAM	Ampicillin	2–4	4
β-LACTAM	Benzylpenicillin	2–4	4
β-LACTAM	Cefacetryl	20–40	125
β-LACTAM	Cefalexin	40–80	100
β-LACTAM	Cefalonium	4–8	20
β-LACTAM	Cefazolin	15–25	50
β-LACTAM	Cefoperazone	1–3	50
β-LACTAM	Cefquinome	8–15	20
β-LACTAM	Cefapirin	6–10	60
β-LACTAM	Ceftiofur	50–70	100
β-LACTAM	Desfuroylceftiofur	50–70	100
β-LACTAM	Cefuroxime	15–25	50
β-LACTAM	Cloxacillin	15–25	30
β-LACTAM	Dicloxacillin	15–20	30
β-LACTAM	Oxacillin	15–25	30
SULFONAMIDE	Sulfadiazine	10–20	100
SULFONAMIDE	Sulfadimethoxine	10–20	100
SULFONAMIDE	Sulfametazine	10–20	100
SULFONAMIDE	Sulfamethoxazole	30–50	100
SULFONAMIDE	Sulfapirydine	10–20	100
SULFONAMIDE	Sulfatiazole	10–20	100
SULFONAMIDE	Sulfacetamide	30–50	100
SULFONAMIDE	Sulfachlorpyridazine	10–20	100
SULFONAMIDE	Sulfadoxine	80–100	100
SULFONAMIDE	Sulfaethoxypyridazine	10–20	100
SULFONAMIDE	Sulfamerazine	20–40	100
SULFONAMIDE	Sulfamethizole	10–20	100
SULFONAMIDE	Sulfamethoxypyridazine	20–40	100
SULFONAMIDE	Sulfaquinoxaline	10–20	100
SULFONAMIDE	Sulfisoxazole	10–20	100
TETRACYCLINE	Chlorotetracycline	40–70	100
TETRACYCLINE	Oxytetracycline	40–70	100
TETRACYCLINE	Tetracycline	5–20	100

(continued on next page)

Table 3 (*continued*)

Method: Delvotest® SP NT			
Chemical Class	Compound	CCβ (ng mL^{-1}) Detection capability	REG. 37/2010/CE MRLs (μg kg^{-1})
TETRACYCLINE	Doxyicline	80–100	Not for use in animals from which milk is produced for human consumption
Method: Bioeasy Milk Antibiotic 3in1			
Chemical Class	**Compound**	**CCβ (ng mL-1) Detection capability**	**REG. 37/2010/CE MRLs (μg kg-1)**
MACROLIDE	Tylosin	10–20	50
MACROLIDE	Tilmicosin	40–50	50
MACROLIDE	Erythromicyn A	3–5	40
LYNCOSAMIDE	Lincomycin	3–6	150

3.2. Antibiotic residue detection in real samples

Overall, among 254 milk samples analysed by using the LC-HRMS method, no antimicrobial residues were detected at a concentration above their MRLs, confirming safety criteria for human consumption as reported in Table 4. Lincomycin residues were observed in 30 samples, representing 11.8% frequency with 17.29 ppb as mean value (0.77 minimum - 54.89 maximum). Among other substances, 3 samples showed oxytetracycline residues respectively at 15.05, 0.82 and 1.59 ppb and 2 showed cefapirin and spyramycin traces (<LOQ). Lincomycin belongs to the lincosamides class that represents a group of antibiotics commonly used in both human and veterinary medicine (Spížek & Řezanka, 2017). Residues of macrolides and lincosamides may remain in food due to their widespread use (Cabizza et al., 2018). Scarce evidence is available in literature concerning lincomycin detection in bovine milk. The most common antibiotic molecules used in cow farms are represented by beta-lactams and tetracycline for mastitis treatments or prevention (Landers, Cohen, Wittum, & Larson, 2012). The predominant source of milk contamination with antibiotics is represented by direct administration but can also occur during milking when the inner surface of a part of the milking machine is rinsed after milking a treated cow milk before an untreated one (Kebede, Zenebe, Disassa, & Tolosa, 2014).

In addition, the presence of low antibiotic levels (below MRL) can lead to the diffusion of antibiotic resistant bacteria. Examining the detected residues by comparing the results obtained from screening tests, only false positive results were obtained. In particular, Delvo and Charm reported 6 and 17 false positive results respectively toward sulfonamide and beta-lactam residues. These frequencies are similar to those found in other research in which the Delvo test was used for antibiotic screening in milk (Bion et al., 2015). A different scenario occurred for the 3easy test since 40 samples were found to be false positive results for lincomycin residues resulting in a 15.74% frequency of false detections. It is important to underline that milk safety assessment implies the use of rapid tests at the farm as well as at a milk processor level to avoid economic losses also due to analytical costs for confirmation techniques. As a consequence, it is fundamental to assess and validate rapid tests able to screen for a high number of antibiotics belonging to different chemical classes in order to set up effective monitoring plans considering both safety and technological issues.

3.3. Impact of antimicrobial residues on cheese-making

In order to investigate the possible impact of antibiotic residues on milk cheese-making, even if present at concentrations below the MRLs, considering the detected residue concentrations, three different lincomicyn concentrations (10, 20, 40 ng g^{-1}) were selected to fortify milk samples previously analysed involved in PDO cheese production. As described in the literature (Hill, 2000), the processing of milk for cheese production depends on several factors including milk composition and quality, auxiliary materials (e.g. starters) and process parameters (e.g., time, temperature, pH). In particular, lincomycin was used as it was revealed in several samples. Research has already been conducted evaluating the impact of antimicrobial substances but oriented to define their distribution in cheese or in by-products in relation to physico-chemical properties. This is important considering that by-products are also utilised in other sectors for human consumption and animal feeding, leading to potential antimicrobial resistance diffusion. However, scarce literature is available on direct influence of residues on bacterial cultures used in cheese production (Katla, Kruse, Johnsen, & Herikstad, 2001).

Analysing the results obtained from pH, acidity and microbiological analyses, it can be seen that the pH trend during the 12 h of acidification decays steadily over time both in the unfortified sample and in those fortified with different concentrations of lincomycin. Specifically, the

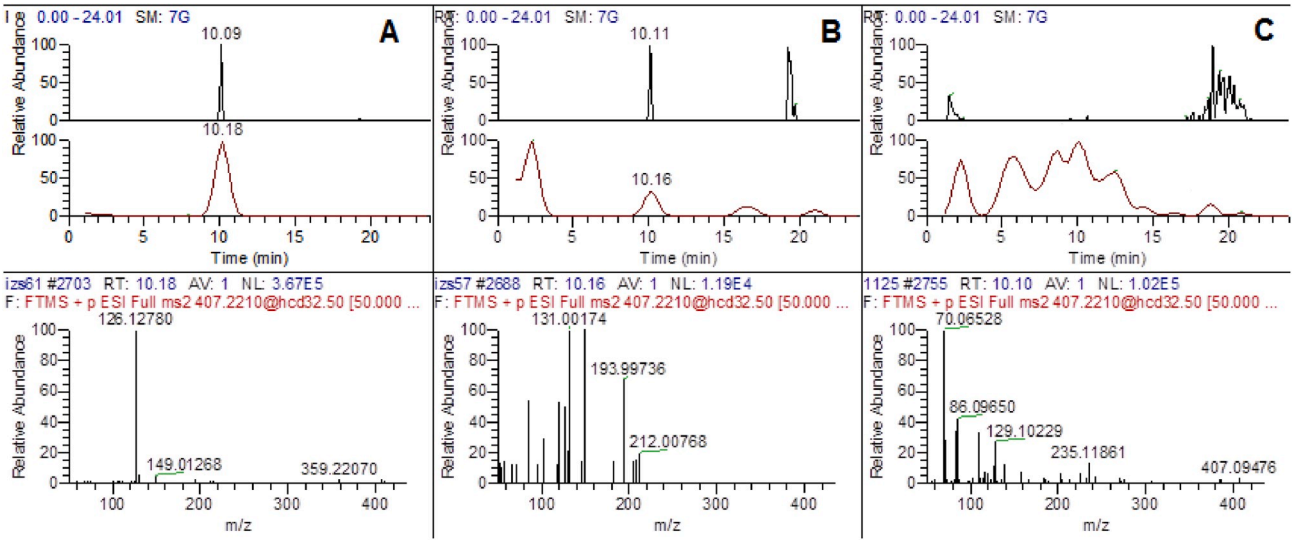

Fig. 2. Extracted parent ion chromatograms from FS and from DIA with the relative fragmentation mass spectra of lincomycin in a positive sample (A), in a sample with an interference where lincomycin is expected to elute (B) and in a negative sample (C).

Table 4
Presence of antibiotic residues in raw milk samples by using LC-HRMS detection (ng mL^{-1}).

Chemical Class	Compound	Mean* (n = 254)	Min-Max	Dev. std (±)
QUINOLON	Enrofloxacin	n.d.**	–	–
QUINOLON	Ciprofloxacin	n.d.	–	–
QUINOLON	Difloxacin	n.d.	–	–
QUINOLON	Danofloxacin	n.d.	–	–
QUINOLON	Levofloxacin	n.d.	–	–
QUINOLON	Lomefloxacin	n.d.	–	–
QUINOLON	Marbofloxacin	n.d.	–	–
QUINOLON	Norfloxacin	n.d.	–	–
QUINOLON	Enoxacin	n.d.	–	–
QUINOLON	Flumequine	n.d.	–	–
QUINOLON	Nadifloxacin	n.d.	–	–
QUINOLON	Oxolinic acid	n.d.	–	–
QUINOLON	Nalidixicacid	n.d.	–	–
β-LACTAM	Amoxicillin	n.d.	–	–
β-LACTAM	Ampicillin	n.d.	–	–
β-LACTAM	Phenoxymethylpenicillin	n.d.	–	–
β-LACTAM	Benzylpenicillin	n.d.	–	–
β-LACTAM	Cefadroxil	n.d.	–	–
β-LACTAM	Cefalexin	n.d.	–	–
β-LACTAM	Cefalonium	n.d.	–	–
β-LACTAM	Cefalothin	n.d.	–	–
β-LACTAM	Cefazolin	n.d.	–	–
β-LACTAM	Cefoperazone	n.d.	–	–
β-LACTAM	Cefquinome	n.d.	–	–
β-LACTAM	Cefapirin	<CCβ	–	–
β-LACTAM	Ceftiofur	n.d.	–	–
β-LACTAM	Desfuroylceftiofur	n.d.	–	–
β-LACTAM	Cloxacillin	n.d.	–	–
β-LACTAM	Dicloxacillin	n.d.	–	–
β-LACTAM	Desacetylcefapirin	n.d.	–	–
β-LACTAM	Nafcillin	n.d.	–	–
β-LACTAM	Oxacillin	n.d.	–	–
β-LACTAM	Piperacillin	n.d.	–	–
MACROLIDE	Tylosin	n.d.	–	–
MACROLIDE	Tilmicosin	n.d.	–	–
MACROLIDE	Oleandomycin	n.d.	–	–
MACROLIDE	Spiramycin	<CCβ	–	–
MACROLIDE	Neospiramycin	n.d.	–	–
MACROLIDE	Kitasamycin	n.d.	–	–
MACROLIDE	Josamycin	n.d.	–	–
MACROLIDE	Tulathromycin	n.d.	–	–
MACROLIDE	Erythromicyn A	n.d.	–	–
SULFONAMIDE	Sulfadiazine	n.d.	–	–
SULFONAMIDE	Sulfadimethoxine	n.d.	–	–
SULFONAMIDE	Sulfadimidine	n.d.	–	–
SULFONAMIDE	Sulfamerazine	n.d.	–	–
SULFONAMIDE	Sulfamethoxazole	n.d.	–	–
SULFONAMIDE	Sulfamonomethoxine	n.d.	–	–
SULFONAMIDE	Sulfapirydine	n.d.	–	–
SULFONAMIDE	Sulfatiazole	n.d.	–	–
SULFONAMIDE	Trimethoprim	n.d.	–	–
TETRACYCLINE	Chlorotetracycline	n.d.	–	–
TETRACYCLINE	Oxytetracycline	5.82	0.82–15.05	8.00
TETRACYCLINE	Tetracycline	n.d.	–	–
TETRACYCLINE	Doxyicline	n.d.	–	–
LYNCOSAMIDE	Lincomycin	17.29	0.77–54.84	22.92
AMPHENICOL	Chloramphenicol	n.d.	–	–
AMPHENICOL	Tiamphenicol	n.d.	–	–
AMPHENICOL	Florfenicol	n.d.	–	–
AMPHENICOL	Florfenicol amine	n.d.	–	–
PLEUROMUTILIN	Tiamulin	n.d.	–	–
PLEUROMUTILIN	Valnemulin	n.d.	–	–
NITROIMIDAZOLE	Dimetridazole	n.d.	–	–
NITROIMIDAZOLE	Ronidazole	n.d.	–	–
NITROIMIDAZOLE	Tinidazole	n.d.	–	–
NITROIMIDAZOLE	Metronidazole	n.d.	–	–

* = ng mL^{-1}, n.d = not detected (<CCα).

samples at 1 h have a pH ranging between 6.39 in B samples, 6.34 in L1, arriving after 12 h at a range between 3.76 in L2 and 4.2 in L3. The pH decay appeared consistent in all samples but with different trends, as shown in Fig. 3, and in particular the most acid pH value (3.76) was found in the unfortified sample at 12h of the acidification process. Moreover, Loftin, Adams, Meyer, and Surampalli (2008) showed that lincomycin remains stable in pH variations and therefore its presence does not directly affect such variations. This result was also confirmed in this study analysing the lincomycin concentration before and after the acidification process (Fig. 3). The three quantities used for initial milk fortification (10, 20 and 40 ng g^{-1}) were then detected and quantified with minimal standard deviation (less than 5%).

The lowering of the pH is very important in the cheese-making process because it increases the activity of enzymes and the speed of coagulation and this phase is of crucial importance especially in hard and long-matured cheeses such as PDO or IGP cheese (Parmigiano Reggiano and Grana Padano) (Lawrence, Heap, & Gilles., 1984; Paquet, Lacroix & Thibault, 2000). In addition, the insufficient lowering of pH can cause early fermentation, supported by clostridia or by yeasts, with formation of large spongy cavities in the cheese (Pecorari, Gambini, Reverberi, & Caroli, 2003). The decay of pH, as described by Paquet et al. (2000), is highly correlated to the acidification process that takes place by lactic acid bacteria, indeed the results obtained in this study show this relation, and, as in pH results, acidity is higher in the unfortified sample (>25°SH/50).

As regards the microbiological aspect the total bacterial count should never exceed a maximum value to reduce the risk of milk quality being compromised in terms of its cheese-making capacity (e.g. altered levels of pH at different stages of production) (Hill, 2000).

At the end of 2 h acidification process, microbiological analyses were also conducted to assess the potential negative impact of lincomycin at ppb levels on bacteria with particular attention towards lactic acid bacteria. As expected, decrease of mesophilic aerobic bacteria number (from 8,26 to 6,86 log CFU/ml^{-1}) was observed, specifically in the unfortified samples well as in the samples fortified with 10 ppb of lincomycin the bacterial growth was higher: 8,26 log CFU/ml^{-1} and 8,11 log CFU/ml^{-1} respectively, compared with the samples fortified with 20 (7,44 log CFU/ml^{-1}) and 40 ppb of lincomycin (6,86 log CFU/ml^{-1}), this due to the antibacterial activity of lincomycin. Also, with regard *Lactobacillus* spp. and lactic streptococci even in this scenario there has emerged a slight decrease of the lactic microflora in fortified samples with different concentrations of lincomycin compared to the unfortified samples, leading to a partial inhibition of the acidification process of milk starter cultures. In particular, the number of *Lactobacillus* spp. decreases from 10,32 log CFU/ml^{-1} in the unfortified samples and 10,20 log CFU/ml^{-1} in the samples fortified with 10 ppb of lincomycin, to 9,50 log CFU/ml^{-1} and 8,92 log CFU/ml^{-1} in the samples fortified with 20 and 40 ppb of lincomycin respectively. While even whit regards lactic streptococci there was a slight decrease from 7,12 log CFU/ml^{-1} (unfortified samples) and 7,20 log CFU/ml^{-1} (samples fortified with 10 ppb of lincomycin) to 6,67 log CFU/ml^{-1} (samples fortified with 20 ppb of lincomycin) and 6,79 log CFU/ml^{-1} (samples fortified with 40 ppb of lincomycin).

The results obtained in this study confirm that milk complies with food safety criteria since the antibiotic residue concentration was lower than the MRL in all samples, but the presence of residues have a negative impact on milk starter cultures and in general on cheese-making, affecting process parameters and in particular lactic microflora. This aspect is critical mainly considering the economic value of PDO cheese and the persistence of antibiotic residues (Duboc & Mollet, 2001). Examining antimicrobial resistance (AMR) phenomena, different studies reported the association of antibiotic-resistant lactobacilli in dairy products. For instance, Ammar et al. (2018) shows the results of antibiotic resistance of *Lactobacillus* spp. cultures isolated from cheese samples. All lactobacillus isolates were found to be resistant to oxacillin (1 μg) and one *Lactobacillus* spp. isolate was also resistant to

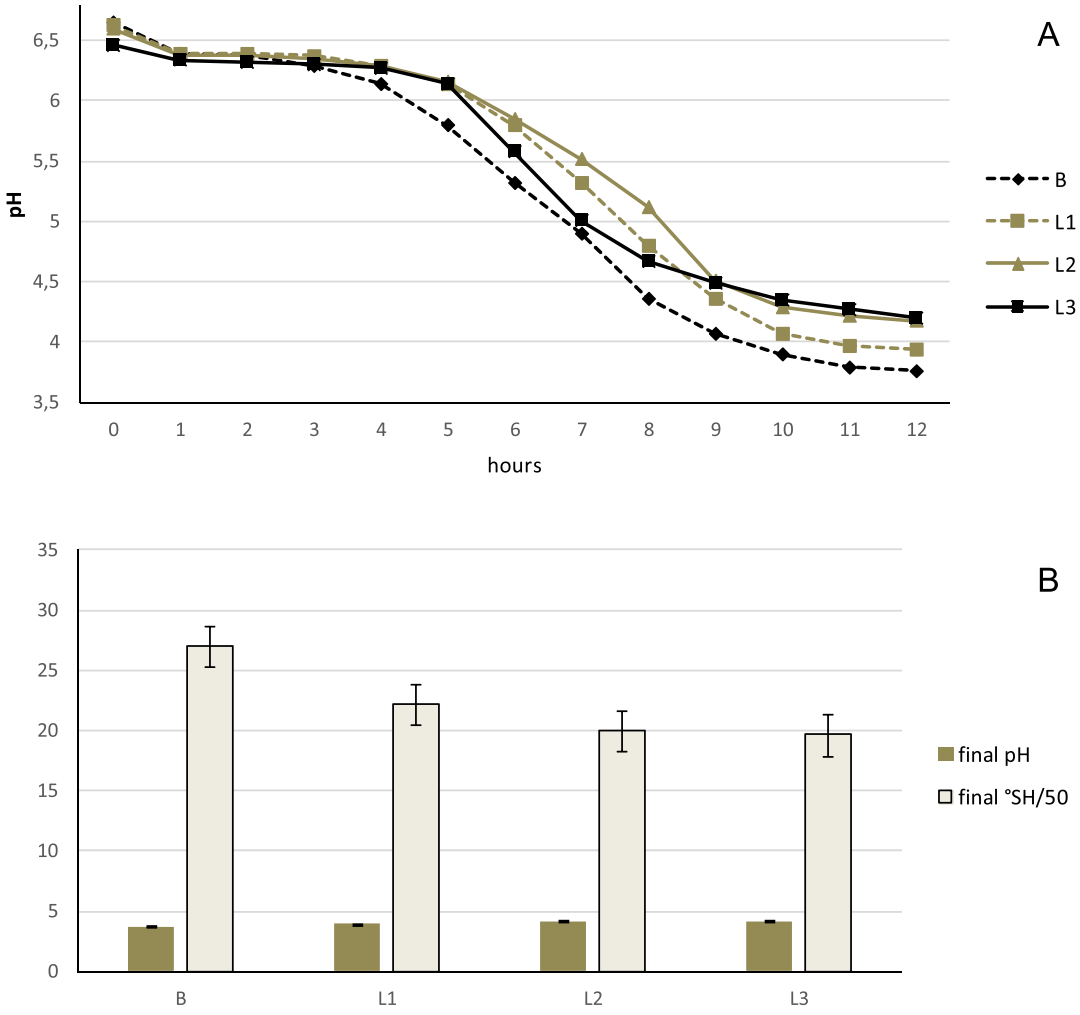

Fig. 3. pH trend during 12 h acidification process (A) and final pH and acidity (B) of milk added with whey starter and fortified at different lincomycin concentrations (blank, B; 10 ppb, L1; 20 ppb L2 and 40 ppb, L3).

streptomycin (10 μg), moreover most of the *Enterococcus* spp. cultures isolated in this study were found to be resistant to lincomycin (10 μg). Another study (Katla et al. 2001) reports the resistance to streptomycin of 188 *Lactobacillus* isolates recovered from Norwegian dairy products.

4. Conclusion

An accurate and sensitive LC–HRMS assay was developed and validated for the simultaneous determination of 68 antibiotics in raw milk with good validation traits to cover broad range of residues belonging to different chemical classes in a complex matrix as milk. The method was then used as confirmatory approach to assess the feasibility of use of 3 rapid screening tests for the rapid detection of antibiotics presence as possible strategies in monitoring plan at farm level. The key performance criterion of a screening method relies on its ability to sort out samples in two baskets, absent vs. present, free vs. suspect, low vs. high, compliant vs. non-compliant, with the highest level of confidence as possible in the results. The 3 tests covered the detection of all residues with an acceptable frequency of false positive results and absence of false negative ones. False negative responses release raw materials and food products that violate the rules of a global business (regulatory limits exceeded) and expose consumers to health risks, ultimately the society to health threats associated to the occurrence of low levels of antibiotics and other residues. 254 raw milk samples involved in PDO cheese production were then analysed to investigate the incidence and concentration of residues. From a safety point of view, the concentrations of detected residues, in particular limited to lincomycin below to its MRL, confirms the safety traits of the raw milk samples investigated since only lincomycin were present in a concentration range from 0.77 to 54.74 ppb. As regards to the influence of revealed residues on cheese-making, a negative effect was demonstrated towards lactic acid bacteria activity with direct consequences for cheese-making since all lincomycin concentration testes (10, 20 and 40 ppb) in cheese making simulation trials have showed a negative role during acidification process of milk. Antimicrobial residues can lead to a partial or complete inhibition of acid production by starter cultures leading to technological and economic impact on the dairy industry as primary consequences. On the other hand, is remaining mandatory the study of the incidence of antibiotic residues in milk sector in order to prevent the antibiotic resistance diffusion also along all food chain by set up best practices also for a correct management of use of antibiotic for animal health.

Declaration of competing interest

There are no conflicts to declare.

CRediT authorship contribution statement

Luca Maria Chiesa: Conceptualization, Project administration, Supervision, Resources. **Lucia DeCastelli:** Data curation, Writing - original draft. **Maria Nobile:** Formal analysis, Validation, Data curation, Writing - review & editing. **Francesca Martucci:** Writing - original draft. **Giacomo Mosconi:** Formal analysis. **Mauro Fontana:** Conceptualization, Methodology. **Marta Castrica:** Formal analysis, Data curation, Writing - original draft. **Francesco Arioli:** Writing - original draft. **Sara Panseri:** Conceptualization, Supervision, Funding acquisition, Writing - original draft.

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