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Research article

# Screening of antibiotic residues in raw bovine milk in Lombardy, Italy: Microbial growth inhibition assay and LC-HRMS technique integration for an accurate monitoring

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ABSTRACT

Antibiotic residues in food of animal origin is a great concern for public health worldwide in terms of antibiotic resistance development, potential allergic reactions and disruption of intestinal flora equilibrium. In this study the presence of antibiotic residues in raw bovine milk samples collected from farms located in Lombardy region in Italy from 2018 to 2022 was assessed in the context of the national milk quality payment system. Samples were screened with microbiological growth inhibition test Delvotest ® SP NT and a very low positivity rate ranging from 0.1% to 0.07% over the four years was determined. A total of 79 positive samples were further analysed by LC-HRMS screening technique to confirm positivity and detect the specific antibiotic compound contaminating the sample. The β-lactam antibiotics resulted to be the most frequently detected, with the penicillin G being the most abundant compound. The data suggested that low levels of antibiotic contamination are consistently maintained over the last four years and the integration of the techniques used in this study is a valuable tool for a deep and precise monitoring of antibiotic residues in milk.

## 1. Introduction

Antibiotics are widely used in food-producing animals for preventive and therapeutic purposes and may result in the contamination of derived food, such as meat, eggs, and milk, with antibiotic residues. Antimicrobial agents' residues could represent a threat for human health triggering allergic reactions [1], disrupting the equilibrium of human intestinal microbiota [2], and contributing to antibiotic resistance dissemination [3,4]. Dairy cattle are frequently exposed to mastitis, mainly contagious bacterial infection with a great impact on the animal health and milk production yields and quality [5]. As a consequence, dairy cattle often undergo pharmacological treatments with broad-spectrum antibiotics from β-lactam, macrolide, quinolone and sulfonamide families to prevent and treat clinical mastitis [6]. Fostered by improper treatment regimens or not observed withdrawal periods, low concentrations of active

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ingredients and their metabolites may accumulate in animal tissues and be secreted with milk, posing a potential risk to consumers [7, 8]. In European Union food contamination with veterinary drugs, including antibacterial substances, is under the strict control since the maximum residue limits (MRLs) in animal-derived food for human consumption are established in the Commission Regulation EU 37/2010 [9]. In Italy, around 12 million tons of raw cow milk is produced every year [10] and Lombardy region accounts for 40–41% of the Italian raw milk production over the last 5 years [11]. Its substantial part is intended for cheese production: for instance, 480.000 tons of PDO (Protected Designation of Origin) cheese were produced in Italy in 2020 [11]. To assure the manufacturing of high-quality dairy products operators are demanding for high-quality antibiotic-free raw milk [12]. Thus, bulk raw milk from each farm in Lombardy region is routinely screened by laboratories included in the regional register of accredited laboratories in the context of milk quality payment system. The latter system was implemented to define the price of supplied milk and, as a consequence, to encourage milk quality improving by the suppliers. Bonuses or penalties are applied to the price based on the monitoring of fat/protein composition, acidity, microbiological parameters, and somatic cells count [13]. Microbial inhibitory substances are also tested to assure the raw milk entering the food chain is free of antibiotic contamination for food safety and technological purposes. Veterinary drug residues can be retained at detectable concentrations in dairy products after skimming, pasteurisation, cheese fermentation and ripening processes [14,15]. While somatic cells count is the reportedly main parameter affecting dairy products processing [16,17], the role of antibiotic residues is not equally well established, although they have been reported to interfere with the starter cultures used for fermented milk products [18,19].

Due to a variability of antibiotic compounds used for dairy cattle treatments, milk screening methods for antibiotic residues need to be able to detect different families of compounds with variety of chemical structures. Microbial growth inhibition methods are widely employed to screen large quantities of milk samples for quality monitoring [20–23]. These tests detect preferentially antibiotic agents from β-lactam family, while the detection capability and sensitivity for aminoglycosides, macrolides and quinolones are significantly lower. Delvotest® SP NT is a rapid screening method based on the growth inhibition of test organism *Bacillus stearothermophilus* var. *calidolactis* by inhibitory substances potentially contaminating milk samples. It is validated for the detection of β-lactam compounds, particularly amoxicillin, ampicillin, cefapirin and penicillin G in raw cow milk [24].

More recently, mass spectrometry based analytical methods, allowing more sensitive and specific qualitative and quantitative multiclass analysis of antibiotic residues, have been developed [25,26]. These methods require sophisticated equipment, highly trained personnel and time-consuming sample extraction and purification resulting in the expensive testing, hardly applicable for screening purposes. However, confirmatory chemical methods application should be applied in order to corroborate the presence of antibiotic residues, and identify the specific agents contaminating the sample.

In this study the routine data collected over the four-years period from January 2018 to June 2022 by the National Reference Centre for Bovine Milk Quality, IZSLER (Istituto Zooprofilattico della Lombardia e dell'Emilia Romagna "Bruno Ubertini") in the context of milk quality payment system from farms located in Lombardy region in Italy were considered. Our aim was to assess the quality of raw milk prior to technological transformation in terms of the incidence of antibiotic contamination and the identification of antimicrobial agents detectable in milk samples. To this purpose milk samples were screened by Delvotest ® SP NT analysis and screen positive samples confirmed by multiclass liquid chromatography-high resolution mass spectrometry (LC-HRMS) screening method providing semi-quantitative identification of the antibiotic compounds more frequently detectable in milk samples.

## 2. Materials and methods

### 2.1. Sample collection

A total of 408.033 bovine milk samples were collected from dairy cattle farms located in Lombardy region in Italy during the years 2018–2022. Sampling was performed from storage tanks by farmers and each sample was representative of the whole herd.

Samples were transferred and stored at 0–4 °C until the analysis had to be performed within 48 h from collection. Microbial growth inhibition test positive samples were stored at −20 °C for further investigation with LC-HRMS technique.

### 2.2. Delvotest ® SP NT screening

#### 2.2.1. Reagents, standards and growth medium

The Delvotest ® SP NT kit for determination of antibiotics was supplied by DSM Food Specialties (Delft, The Netherlands). The following standards were produced by National Reference Centre for Bovine Milk Quality, IZSLER (Brescia, Italy): inhibitory substances-free milk, penicillin standard reference solution and sulfadiazine standard reference solution.

A commercially available UHT whole milk was purchased and used to prepare the inhibitory substances-free milk. First, the UHT milk was tested for absence of antibiotics and determination of fat, proteins, lactose, total bacterial count, somatic cells count and urea. Afterwards, milk was prepared for freeze drying, 12 g for each vial. After freeze drying, 5% of the total number of vials was tested for absence of antibiotics, while the remaining were stored at 6 ± 4 °C for up to 10 years. Vials were replenished with 10.8 g of distilled water. Sodium azide (Merck KGaA, Darmstadt, Germany) was added to part of the replenished vials, as the inhibitory substances-free milk with preservative substance had to be tested in every analysis. All replenished vials were sub-divided in 1 ml vial each and used within a day or stored for a year at a temperature of −24 ± 6 °C.

Both Standard reference solutions of penicillin and sulfadiazine were prepared using a commercially available UHT semi-skimmed milk, which was tested as described for the inhibitory substances-free milk. Penicillin standard was prepared by adding a penicillin G Potassium 97% purity (Merck KGaA, Darmstadt, Germany) to the UHT semi-skimmed milk to reach a final concentration of 4 μg/kg,

**Table 1**
Molecular formulas, retention times, adducts and monoisotopic exact masses of 62 analytes and deuterated internal standards used for LC-HRMS acquisition.

| Compound family | Reference supplier | Analyte | Molecular formula | RT (min) | Adduct | Exact mass *(m/z)* |
|---|---|---|---|---|---|---|
| Amphenicols | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Florfenicol | C12H14Cl2FNO4S | 10.1 | $[M+H]^+$ | 358.0077 |
| | | Florfenicol amine | C10H14FNO3S | 2.9 | $[M+H]^+$ | 248.0751 |
| | | Thiamphenicol | C12H15Cl2NO5S | 8.4 | $[M+H]^+$ | 356.0121 |
| | TRC Inc. (Toronto, Canada) | Florfenicol-d3 | C12H11D3Cl2FNO4S | 10.1 | $[M+H]^+$ | 361.0266 |
| β-lactams | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Amoxicillin | C16H19N3O5S | 6.3 | $[M+H]^+$ | 366.1118 |
| | | Ampicillin | C16H19N3O4S | 9.9 | $[M+H]^+$ | 350.1169 |
| | | Cloxacillin | C19H18ClN3O5S | 16.6 | $[M+Na]^+$ | 458.0548 |
| | HPC-standards GmbH (Borsdorf, Germany) | Dicloxacillin | C19H17Cl2N3O5S | 17.3 | $[M+Na]^+$ | 492.0158 |
| | | Oxacillin | C19H19N3O5S | 16.3 | $[M+Na]^+$ | 424.0938 |
| | | Nafcillin | C21H22N2O5S | 17.4 | $[M+Na]^+$ | 437.1142 |
| | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Penicillin G | C16H18N2O4S | 15.0 | $[M+Na]^+$ | 357.0880 |
| | | Penicillin V | C16H18N2O5S | 16.3 | $[M+Na]^+$ | 373.0829 |
| | | Cefoperazone | C25H27N9O8S2 | 10.3 | $[M+H]^+$ | 646.1497 |
| | | Cefalexin | C16H17N3O4S | 9.5 | $[M+H]^+$ | 348.1013 |
| | | Cefquinome | C23H24N6O5S2 | 7.9 | $[M+2H]^{2+}$ | 265.0698 |
| | TRC Inc. (Toronto, Canada) | Desacetylcefapirin | C15H15N3O5S2 | 4.9 | $[M+H]^+$ | 382.0526 |
| | | Cefapirin | C17H17N3O6S2 | 7.3 | $[M+H]^+$ | 424.0632 |
| | | Cefazoline | C14H14N8O4S3 | 9.8 | $[M+H]^+$ | 455.0373 |
| | Merck KGaA (Darmstadt, Germany) | Ceftiofur | C19H17N5O7S3 | 12.9 | $[M+H]^+$ | 524.0363 |
| | | Cefalonium | C20H18N4O5S2 | 8.3 | $[M+H]^+$ | 459.0791 |
| | TRC Inc. (Toronto, Canada) | Penicillin G-d7 | C16H11D7N2O4S | 15.0 | $[M+Na]^+$ | 364.1319 |
| | | Penicillin V-d5 | C16H13D5N2O5S | 16.2 | $[M+Na]^+$ | 378.1142 |
| | | Amoxicillin-d4 | C16H15D4N3O5S | 6.3 | $[M+H]^+$ | 370.1369 |
| | | Ampicillin-d5 | C16H14D5N3O4S | 9.9 | $[M+H]^+$ | 355.1483 |
| | | Cefuroxime-d3 | C16H13D3N4O8S | 9.6 | $[M+Na]^+$ | 450.0769 |
| Quinolones | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Nalidixic acid | C12H12N2O3 | 14.8 | $[M+H]^+$ | 233.0920 |
| | | Oxolinic acid | C13H11NO5 | 12.8 | $[M+H]^+$ | 262.0710 |
| | | Sarafloxacin | C20H17F2N3O3 | 10.3 | $[M+H]^+$ | 386.1311 |
| | | Danofloxacin | C19H20FN3O3 | 9.7 | $[M+H]^+$ | 358.1561 |
| | | Flumequine | C14H12FNO3 | 15.1 | $[M+H]^+$ | 262.0874 |
| | Merck KGaA (Darmstadt, Germany) | Difloxacin | C21H19F2N3O3 | 9.9 | $[M+H]^+$ | 400.1467 |
| | | Enrofloxacin | C19H22FN3O3 | 9.6 | $[M+H]^+$ | 360.1718 |
| | | Levofloxacin | C18H20FN3O4 | 9.0 | $[M+H]^+$ | 362.1511 |
| | | Marbofloxacin | C17H19FN4O4 | 8.5 | $[M+H]^+$ | 363.1463 |
| | | Norfloxacin | C16H18FN3O3 | 9.3 | $[M+H]^+$ | 320.1405 |
| | | Ciprofloxacin | C17H18FN3O3 | 9.6 | $[M+H]^+$ | 332.1405 |
| | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Enrofloxacin-d5 | C19H17D5FN3O3 | 9.6 | $[M+H]^+$ | 365.2032 |
| Diaminopirimidins | Merck KGaA (Darmstadt, Germany) | Trimethoprim | C14H18N4O3 | 8.5 | $[M+H]^+$ | 291.1451 |
| Lincosamides | HPC-standards GmbH (Borsdorf, Germany) | Lincomycin | C18H34N2O6S | 8.5 | $[M+H]^+$ | 407.2210 |
| Macrolides | TRC Inc. (Toronto, Canada) | 3-*O*-acethyltylosin | C48H79NO18 | 16.0 | $[M+H]^+$ | 958.5370 |
| | | Gamitromycin | C40H76N2O12 | 12.9 | $[M+H]^+$ | 777.4571 |
| | | Neospiramycin I | C36H62N2O11 | 11.3 | $[M+CH_3OH+2H]^{2+}$ | 366.2381 |
| | | Tulathromycin | C41H79N3O12 | 9.6 | $[M+3H]^{3+}$ | 269.5295 |
| | | Tilvalosin | C53H87NO19 | 17.8 | $[M+H]^+$ | 1042.5945 |
| | | Spiramycin I | C43H74N2O14 | 12.0 | $[M+CH_3OH+2H]^{2+}$ | 438.2774 |
| | Pharm A2S (Saint Jean d'Illac, France) | Tildipirosin | C41H71N3O8 | 7.3 | $[M+3H]^{3+}$ | 245.5153 |
| | Merck KGaA (Darmstadt, Germany) | Tilmicosin | C46H80N2O13 | 13.6 | $[M+2H]^{2+}$ | 435.2903 |
| | Pharm A2S (Saint Jean d'Illac, France) | Erythromycin A | C37H67NO13 | 15.8 | $[M+H]^+$ | 734.4685 |
| | Dr. Ehrenstorfer GmbH (Augsurg, Germany) | Tylosin A | C46H77NO17 | 15.7 | $[M+CH_3OH+H]^+$ | 948.5526 |
| | TRC Inc. (Toronto, Canada) | Spiramycin I-d3 | C43H71D3N2O14 | 12.0 | $[M+H]^+$ | 439.7868 |
| Pleuromutilins | Dr. Ehrenstorfer GmbH (Augsurg, Germany) | Thiamulin | C28H47NO4S | 15.3 | $[M+H]^+$ | 494.3299 |
| | Pharm A2S (Saint Jean d'Illac, France) | Valnemulin | C31H52N2O5S | 17.4 | $[M+H]^+$ | 565.3670 |
| Rifamicins | Merck KGaA (Darmstadt, Germany) | Rifaximin | C43H51N3O11 | 18.8 | $[M+H]^+$ | 786.3596 |
| Sulfonamides | Dr. Ehrenstorfer GmbH (Augsurg, Germany) | Sulfachlorpirydazine | C10H9ClN4O2S | 9.6 | $[M+H]^+$ | 285.0208 |
| | | Sulfadiazine | C10H10N4O2S | 6.5 | $[M+H]^+$ | 251.0597 |
| | | Sulfamerazine | C11H12N4O2S | 7.9 | $[M+H]^+$ | 265.0754 |

(*continued on next page*)

**Table 1** (*continued*)

| Compound family | Reference supplier | Analyte | Molecular formula | RT (min) | Adduct | Exact mass *(m/z)* |
|---|---|---|---|---|---|---|
| | | Sulfamethazine | C12H14N4O2S | 9.0 | $[M+H]^+$ | 279.0910 |
| | | Sulfamonomethoxine | C11H12N4O3S | 10.0 | $[M+H]^+$ | 281.0703 |
| | | Sulfapyridine | C11H11N3O2S | 7.4 | $[M+H]^+$ | 250.0645 |
| | | Sulfaquinoxaline | C14H12N4O2S | 12.6 | $[M+H]^+$ | 301.0754 |
| | | Sulfathiazole | C9H9N3O2S2 | 7.1 | $[M+H]^+$ | 256.0209 |
| | Merck KGaA (Darmstadt, Germany) | Sulfadimethoxine | C12H14N4O4S | 12.2 | $[M+H]^+$ | 311.0809 |
| | | Sulfamethoxazole | C10H11N3O3S | 9.8 | $[M+H]^+$ | 254.0594 |
| | Dr. Ehrenstorfer GmbH (Augsburg, Germany) | Sulfadimethoxine-d6 | C12H8D6N4O4S | 12.2 | $[M+H]^+$ | 317.1185 |
| Tetracyclines | Dr. Ehrenstorfer GmbH (Augsurg, Germany) | 4-Epichlortetracycline | C22H23ClN2O8 | 10.4 | $[M+H]^+$ | 479.1216 |
| | | 4-Epioxytetracycline | C22H24N2O9 | 9.2 | $[M+H]^+$ | 461.1555 |
| | | 4-Epitetracycline | C22H24N2O8 | 8.4 | $[M+H]^+$ | 445.1605 |
| | | Chlortetracycline | C22H23ClN2O8 | 10.8 | $[M+H]^+$ | 479.1216 |
| | | Doxycycline | C22H24N2O8 | 12.9 | $[M+H]^+$ | 445.1605 |
| | | Oxytetracycline | C22H24N2O9 | 9.5 | $[M+H]^+$ | 461.1555 |
| | | Tetracycline | C22H24N2O8 | 9.4 | $[M+H]^+$ | 445.1605 |
| | TRC Inc. (Toronto, Canada) | Tetracycline-d6 | C22H18D6N2O8 | 9.4 | $[M+H]^+$ | 451.1982 |

divided in vials at 12 g each and freeze-dried. Sulfadiazine sodium 99.8% purity (Merck KGaA, Darmstadt, Germany) was used for the sulfadiazine standard, added to the UHT semi-skimmed milk at a 100 μg/kg concentration. Afterwards, 12.5 g per vial were measured for freeze-drying. Before lyophilization all newly contaminated UHT milk was tested for presence of antibiotics. After freeze drying 5% of the vials were tested for presence of antibiotics, while the remaining were stored at 6 ± 4 °C for up to 10 years. The standards were replenished with different quantity of distilled water (10.7 g for penicillin, 11.36 g for sulfadiazine) to reach the pre-lyophilization antibiotic concentration. Sodium azide was added to part of the replenished vials, as standards with preservative substance had to be tested in every analysis. All replenished vials were sub-divided in 1 ml vial each and used within a day or stored for a year at a temperature of −24 ± 6 °C.

The medium was part of the Delvotest SP-NT kit and consisted of a solid and buffered agar gel, including all required nutrients, spores of test organism *Bacillus stearothermophilus* var. *calidolactis*, and bromocresol purple as pH indicator.

#### *2.2.2. Delvotest ® SP NT analysis*

Milk samples were carefully mixed upside down before testing, avoiding formation of air bubbles or foam. A SKALAR SP2000 automatic distribution system (Breda, The Netherlands) was used for automatic sample inoculation of 100 μl in each well. Dosing tubes and pipetting needles were rinsed with washing solution after every sample inoculation. Negative controls consisted of inhibitory substances-free milk both with and without sodium azide. Penicillin standard and sulfadiazine standard, were used as positive controls, as both tested with and without the addition of sodium azide. All controls were added manually at 100 μl each in specific wells after sample inoculation. The plate was covered with adhesive foil and incubated at 64 ± 1 °C for about 2:45 h in water bath, until negative controls turned yellow. As a matter of facts, negative controls had to be yellowish to be considered valid, as *Bacillus stearothermophilus* was able to grow. Positive controls had a purple/blue colour in each well. Visual interpretation of samples allowed to identify yellowish colour as a negative result. Positive samples displayed a purple/blue colouring, while a partial or irregular coloration was considered as uncertain.

### *2.3. LC-HRMS qualitative screening*

#### *2.3.1. Chemicals and reagents*

Reference molecules and deuterated internal standards with corresponding suppliers used for this study are listed in Table 1. HPLC grade acetonitrile, formic acid and HPLC grade methanol were purchased from Carlo Erba Reagents Srl (Milan, Italy). EDTA disodium salt dihydrate and ammonium acetate powders were obtained from Merck KGaA (Darmstadt, Germany).

#### *2.3.2. Standards preparation*

All individual reference molecules were prepared at 100 μg/ml concentration, with the exception for sulfonamides family that was stocked at the concentration of 500 μg/ml. Amphenicols, macrolides, sulfonamides, tetracyclines, trimetoprim, lyncomicin, and rifaximin stock solutions were prepared in HPLC grade methanol solution, while for quinolones HPLC grade methanol/water (80:20 v/v) solution was used. HPLC grade water/acetonitrile (75:25 v/v) was used to prepare β-lactams solution. Internal standard stock solutions were prepared at 100 μg/ml concentration in water/HPLC grade acetonitrile solution (75:25 v/v) (amoxicillin-d4, ampicillin-d5, penicillin V-d5, penicillin G-d7, cefadroxil-d4, cefuroxime-d3) or in HPLC grade methanol (enrofloxacin-d5, florfenicol-d3, spiramycin I-d3, sulfadimetoxine-d6, tetracycline-d6). Stock solutions were stored at −24 ± 6 °C, with the exception of sulphonamides and trimethoprim, stored at 5 ± 3 °C. The stability of the stock solutions was assessed at appropriate temperature and ranged between 12 and 48 months for all the analytes with the exception of 4-epioxytetracycline, which was stable only for one month. Pool solutions of standard analytes were assembled at proper concentrations to facilitate fortified samples preparation and stored at −24 ± 6 °C up to

one month.

*2.3.3. Sample preparation*

Each positive or uncertain sample from Delvotest ® SP NT analysis was thawed and 5 ± 0.1 g of milk sample were weighed, placed in the 15 ml polypropylene tube, and internal standard pool solutions were added to reach the final concentration of 0.05 μg/mL for each analyte. Bovine milk, previously confirmed as antibiotic-free, was used as blank control. The positive control was prepared by fortification of antibiotic-free milk with analyte pool solutions at CCβ (detection capability) concentration, which was 2 μg/kg for penicillin G and ampicillin, 3 μg/kg for amoxicillin, and 10 μg/kg for all other analytes. For sample extraction, centrifugation step was performed at 4000 rpm for 10 min to separate and discard the fat phase and each sample was treated with 500 μl of EDTA 0.05 M and 8 ml of acetonitrile. After horizontal stirring for 20 min, samples were placed in an ultrasonic bath for 10 min, following by the second centrifugation step at 4000 rpm for 10 min at 4 ± 2 °C. Supernatant liquid was transferred to a fresh 15 ml tube and diluted to 15 ml volume. After vortex agitation a third centrifugation step completed the defatting process and 3 ml of supernatant liquid were dried by nitrogen evaporation. Dry samples were collected with 200 μl of ammonium acetate 0.2 M, placed into the 1.5 ml polypropylene tubes, centrifuged at 13,000 rpm for 20 min at 4 ± 2 °C and transferred to glass vials for LC-HRMS analysis. Blank and fortified samples were prepared in the same conditions.

*2.3.4. LC-HRMS analysis*

A volume of 10 μl was injected in the LC-HRMS system, Thermo Fischer Scientific (San Jose, CA, USA) Accela HPLC connected to the LTQ Orbitrap. Poroshell 120 EC-C18 chromatographic column (100 mm × 3 mm, 2.7 μm, Agilent) protected by the precolumn (Poroshell 120 EC-C18 100 mm × 3 mm, 2.7 μm, Agilent) was used for the separation of analytes. Two mobile phases were used, HPLC grade methanol and HPLC formic acid 0.1% aqueous solution. A binary gradient was used (Table 2), sample flow was constant at 0.25 ml/min.

The compound ionization was performed in Heated Electrospay Ionization (HESI), positive polarization mode and the ionization parameters were: heater temperature 320 °C, ion transfer capillary temperature 300 °C, sheath gas (nitrogen) pressure 35 a.u., auxiliary gas (nitrogen) pressure 15 a.u., ion sweep gas pressure 0 a.u., ion spray voltage 3000 V, capillary voltage: 26 V, tube lens: 70 V. Main acquisition parameters are listed in Table 2. The CCβ values were 10 μg/kg for all the analytes with the exception for ampicillin (2 μg/kg), penicillin G (2 μg/kg) and amoxicillin (3 μg/kg).

Linearity in matrix was tested with a three-point curve corresponding to the concentrations of 1 μg/kg, 5 μg/kg and 10 μg/kg for all the analytes. An estimated concentration of detected antibiotic compounds was provided by the point-to-point calculation, as a ratio between the peak area of the target sample and the peak area of the spiked sample at CCβ level. The recoveries were checked for all the analytes comparing the peak area of each compound in the sample fortified at CCβ level with the peak area in matrix, where the reference analytes were added immediately prior to injection.

The LC-HRMS qualitative screening method was validated following the European Decision 657/2002 [27] which regulates the performance of analytical methods for pharmacological residues determination in animal-derived food. It is worth notice here that Decision 657/2002 has been recently repealed with EU regulation 808/2021 [28]. To assess the specificity and the detection capability (CCβ), 20 repeats of blank samples and samples fortified with each analyte at the concentration corresponding to the MRL (ampicillin at 2 μg/kg, penicillin G at 2 μg/kg, amoxicillin at 3 μg/kg, and the remaining analytes at 10 μg/kg) were analysed. In all the fortified samples the presence of the added analyte was determined. No interferences were identified for each analyte determination.

## 3. Results and discussion

Delvotest ® SP NT is a commercial kit with a validated sensitivity and selectivity for four β-lactam antibiotic compounds. Precisely, according to the manufacturer's declaration, the sensitivity (the concentration for which 95% of the samples analysed are positive) for amoxicillin is 2.5 μg/kg, 3.0 μg/kg for ampicillin, 1.5 μg/kg for penicillin G and 5.8 μg/kg for cefapirin. We also evaluated the test sensitivity for 17 antibiotic compounds from different families as reported in Table 3. The test resulted to be highly responsive to the analytes from β-lactam family, while macrolides and other compounds displayed low sensitivity profiles at the concentrations above the MRLs. Delvotest ® SP NT underwent the interlaboratory proficiency testing over the years, where 4 (3.1%) non-conforming results were identified from a total of 131 samples. Other authors provided validation data on Delvotest ® SP NT and Le Breton et al. [20] detected penicillin, cloxacillin, sulfamethazine, sulfadiazine, cephalexin and gentamicin at or below the MRLs.

During the four years period 408.033 milk samples were analysed. Delvotest® SP NT screening identified a total of 364 screen

**Table 2**
HPLC gradient elution program.

| Time (min) | Methanol HPLC grade (%) | Formic acid water solution 0.1% HPLC grade (%) |
|---|---|---|
| 0 | 5 | 95 |
| 1 | 5 | 95 |
| 20 | 95 | 5 |
| 25 | 95 | 5 |
| 26 | 5 | 95 |
| 30 | 5 | 95 |

**Table 3**
Sensitivity for different antibiotics analysed by Delvotest ® SP NT in milk. Sensitivity is determined by visual reading of colour change. MRLs refer to values fixed by EU Regulation 37/2010.

| Analyte | Tested concentrations (μg/kg) MRLs are indicated in bold | Sensitivity (μg/kg) |
|---|---|---|
| ***β-lactams*** | | |
| Amoxicillin | 6 **4** 3 1.5 1.2 0.6 0.3 | 0.6 |
| Ampicillin | 6 **4** 3 1.5 1.2 0.6 0.3 | 3 |
| Penicillin G | 6 **4** 3 1.5 1.2 0.6 0.3 | 1.2 |
| Cloxacillin | 60 **30** 20 10 5 2.5 1.5 | 5 |
| Dicloxacillin | 60 **30** 20 10 5 2.5 1.5 | 20 |
| Nafcillin | 60 **30** 20 10 5 2.5 1.5 | 5 |
| Oxacillin | 60 **30** 20 10 5 2.5 1.5 | 10 |
| Cefalexin | 150 **100** 90 80 70 60 50 | <50 |
| Cefapirin | 15 **10** 9 8 7 6 5 | <5 |
| Cefazolin | 100 **50** 25 10 5 2.5 1 | 2.5 |
| ***Tetracyclines*** | | |
| Doxycycline* | 150 100 90 80 70 60 50 | 150 |
| ***Sulfamides*** | | |
| Sulfamonomethoxine | 150 **100** 90 80 70 60 50 | <50 |
| ***Macrolides*** | | |
| Erythromycin | 60 **40** 30 15 12 6 3 | >60 |
| Spiramycin | 300 200 100 50 20 10 5 | >300 |
| Tylosin | 100 **50** 25 10 5 2.5 1.2 | >100 |
| ***Others*** | | |
| Thiamfenicol | 100 **50** 25 10 5 2.5 1.2 | >100 |
| Trimethoprim | 200 100 80 **50** 40 25 10 | 80 |
| Lincomycin | 450 300 **150** 100 50 25 10 | 450 |

*Not for use in animals from which milk is produced for human consumption.

positive samples resulted in a 0.08% positivity rate. The annual rate ranged from 0.1% to 0.07%, showing stable and slightly decreasing trend (Table 4). In the context of European scenario these data are in line with the annual reports produced by EFSA, where 0.04%, 0.12%, 0.07% of non-compliant samples for antibacterial substances were reported respectively for 2018, 2019 and 2020 in the context of the national monitoring plans according to the Directive 96/23/EC [29–31]. These data strongly suggest that in terms of antibiotic contamination the quality of milk conferred in Lombardy region maintains elevated standards. This could presumably be the result of European Union legislation on the veterinary drug use directed to antimicrobial resistance control in the food chain combined to the strict territorial controls applied by the state in terms of food safety and elevated standards required for dairy products production. Bilandžić et al. [32] reported similar situation in Croatia, where the legislation on the veterinary drug residues in food of animal origin is aligned to the European requirements, and the incidence of positive samples in the context of official antibiotic residues monitoring was 0.69% during 3-year period from 2008 to 2010. In contrast, a higher percentage of positive results (6.11%) was reported by Rama et al. [33] in Kosovo, where no monitoring programs for drug residues in milk were implemented at the time of testing period (2009–2010), and by Grădinaru et al. [34] in Romania where from 2875 samples 124 (4.45%) tested positive for antibiotic residue screening during 2006–2009 period. However, in the last decade studies on the presence of antibiotic residues in European countries are scarce and the available reports prevalently come from extra European contexts, where the data on the presence of antibiotic residues in milk display different scenarios [35].

Although the microbial growth inhibition test Delvotest ® SP NT is a reliable validated screening tool, false positive or uncertain results with not clear or irregular coloration have been described. False positive results have been correlated to multiple factors, such as the presence of high somatic cell counts deriving from mastitis infections [36,37] and increased levels of lysozyme and lactoferrin [38]. Some concerns have been raised about the high occurrence of false negative results from Delvotest ® SP NT screening. Chiesa et al. [18] reported 68% of false negative screening responses in milk from cows previously treated with antibiotics by parallel testing with highly sensitive LC-HRMS analysis, while non or only a 1.7–4.9% ranges of false negative results have been detected in validation studies for specific molecules (nafcillin, oxytetracycline, tetracycline and rifaximin) for the improved version Delvotest ® T [39]. In

**Table 4**
Number of screen positive and screen negative results of Delvotest® SP NT screening on milk samples during the routine analysis. Percentages are indicated in brackets.

| Year | Routine samples | | |
|---|---|---|---|
| | Screen positive | Screen negative | Total screened by Delvotest® SP NT |
| 2018 | 101 (0.11%) | 95,857 (99.89%) | 95,958 (100%) |
| 2019 | 94 (0.10%) | 94,544 (99.90%) | 94,638 (100%) |
| 2020 | 67 (0.08%) | 84,757 (99.92%) | 84,824 (100%) |
| 2021 | 69 (0.08%) | 88,574 (99.92%) | 88,643 (100%) |
| 2022* | 33 (0.07%) | 43,937 (99.93%) | 43,970 (100%) |

*January–June 2022.

addition to the described limitations of the microbiological technique, the identity of the specific antibiotic residues in the sample remains unknown and a confirmatory analytical method should be performed for a single residue identification.

During the routine milk testing not all the screen positive samples were conserved for further LC-HRMS analysis as the collected quantity of the sample not always matched the demand for the analysis exceeding the programmed milk quality system testing.

Therefore, 79 Delvotest ® SP NT positive samples and 21 uncertain samples have been tested by multiclass LC-HRMS screening technique validated for the detection of 62 antibiotic compounds. LC-HRMS method applied in this study is a semi-quantitative high-resolution screening analysis based on the exact mass ion identification of the analytes and provides only an estimated quantification of the antimicrobial compounds in the sample. We confirmed that 48 (60.8%, n = 79) samples contained at least one antibiotic residue and identified that 6 (28.6%, n = 21) of uncertain samples had detectable levels of β-lactams (Table 5). Two samples contained multiple residues, particularly in one sample ampicillin and dicloxacillin were detected at 28 μg/kg and 13 μg/kg respectively; cefapirin and desacetylcefapirin combined to the lyncomicin residues were detected in one sample at the concentrations below MRLs (12 μg/kg, 11 μg/kg and 12 μg/kg respectively). The single antibiotic could not exceed the MRLs defined by the authorities but the combination of very low concentrations of different compounds could represent an increased risk in terms of food safety. LC-HRMS method detected traces of antibiotic molecules in further 20 samples (17 for screen positive samples and 3 for uncertain), where the residue could be identified but the concentration could not reach the limit of detection levels of the method and therefore they were not considered as confirmed. In the group of confirmed samples 28 residues would exceed the MRLs, (Table 5) with 2 samples displaying estimated concentrations 5-fold above the permitted levels, particularly 324 μg/kg for rifaximin and 28 μg/kg for ampicillin. In the group of 6 uncertain samples, which displayed positivity in the mass-spectrometry analysis, 2 samples reached the estimated concentration of penicillin G around 5 μg/kg which is very close to the MRLs value for the compound (4 μg/kg). However, no concentrations highly exceeding established MRLs have been detected in uncertain samples.

The most prevalent compound was penicillin G, which was detected in 22 screen positive samples. Compounds from β-lactam family represented the majority of positive samples and were detected in the total of 45 samples. It is not surprising because of the large use of β-lactams (penicillins, 1st and 2nd generation cephalosporins) for intramammary infusion treatment of bovine mastitis and the specificity and sensitivity of Delvotest ® SP NT for β-lactam family, particularly for penicillin G, amoxicillin, ampicillin and cefapirin as discussed above. These results are in line with data previously published by Rama et al. [33] that confirmed the presence of β-lactam residues in 32 of 55 samples resulted positive for screening test. In 2003 Ghidini et al. [40] confirmed 31 of 53 Delvotest ® SP NT positive samples for beta-lactams by LC-MS/MS methodology.

The most abundant not β-lactam compound was rifaximin (6 positive samples), a naphthalene-ring ansamycin family antibiotic [41]. Interestingly, a survey on the antimicrobial drugs use in dairy farms conducted by Serraino et al. [42] in Emilia-Romagna region in Northern Italy identified rifaximin as the most used antimicrobial agent not belonging to the β-lactam family.

Rifaximin is not able to cross cell membranes and the oral absorption of this drug is lower than 1%. Due to its properties it is usually used for reproductive system disorders in cows and to locally treat mastitis infections in dry periods [41]. One study from Liu et al. [43] suggested that no rifaximin residues are detectable in milk after 6 h from treatment and no post-treatment withdrawal periods are required for this antibiotic agent. There are no clear data on the sensitivity of Delvotest ® SP NT to rifaximin, although a study from Bion et al. [39] confirmed rifaximin residues detection at 60 μg/kg (MRL concentration). The reason why milk samples containing low levels of rifaximin (11–17 μg/kg as confirmed by LC-HRMS analysis) could be detected as positive is probably the association with cefacetrile, a first-generation cephalosporin drug, in commercially available formulations [42]. Cefacetrile is not a part of the antibiotic compounds screened by the applied LC-HRMS method and therefore its presence could not be confirmed.

## 4. Conclusions

In this work we report that the contamination rate of the local bovine milk during the four years monitoring period from 2018 to

**Table 5**
Antibiotic residues identified by LC-HRMS screening analysis on the positive and uncertain Delvotest ® SP NT results.

| Antibiotic compound | MRL (μg/kg) | Number of positive samples (LC-HRMS) | |
|---|---|---|---|
| | | > MRL (maximum detected level in μg/kg) | <MRL |
| Amoxicillin | 4 | 4 (15) | 1 |
| Ampicillin | 4 | 3 (28) | 2 |
| Penicillin G | 4 | 13 (26) | 9 |
| Cloxacillin | 30 | – | 3 |
| Dicloxacillin | 30 | – | 1 |
| Ceftiofur | 100 | – | 1 |
| Cefalonium | 20 | – | 1 |
| Cefapirin | 60[1] | 1 (121) | 2 |
| Desacetylcefapirin | 60[1] | 1 (176) | 2 |
| Cefoperazone | 50 | 3 (150) | 1 |
| Cefazoline | 50 | – | 1 |
| Lincomycin | 150 | – | 3 |
| Rifaximin | 60 | 2 (324) | 4 |
| Oxytetracyclin | 10 | 1 (236) | – |

[1]Sum of cefapirin and desacetylcefapirin.

2022 was 0.08% as detected by Delvotest® SP NT technique. Mass spectrometry analysis on screen positive milk samples determined that 60.8% had detectable levels of one or more antibiotic residues. The β-lactam antibiotics resulted to be the most frequently detected, with the penicillin G being the most abundant compound. These data suggested that low levels of antibiotic contamination were consistently maintained over the last four years and the integration of the techniques used in this study could be a valuable tool for a deep and precise monitoring of antibiotic residues in milk. The milk quality payment system is a very powerful tool in terms of monitoring milk composition and contamination due to a high-performance screening program allowing to analyse around 400 milk samples from regional farms per day and therefore providing a substantial amount of surveillance data. However, the confirmation step with mass spectrometry-based techniques is important, particularly for the determination of the specific compound contaminating the sample and its concentration, as the milk contaminated with above the MRLs concentrations of antibiotic residues should be reported to the authorities that will reach for the decision of withdrawal from the market. Mass spectrometry-based techniques alone today cannot be applied for such a numerous screening procedure due to elevated costs and time-consuming sample preparation protocols.

The identification of specific antibiotic compounds in bovine milk is crucial as a contribution for implementation of European programs of antimicrobial resistance dissemination control in the animal-derived food. Therefore, an integration of two analytical approaches could be a valuable tool to assure an accurate large-scale monitoring system of specific antibiotic compounds.

## Author contribution statement

**Elena Butovskaya:** Performed the experiments; Analysed and interpreted the data; Wrote the paper.
**Lorenzo Gambi:** Performed the experiments; Wrote the paper.
**Alice Giovanetti:** Performed the experiments.
**Giorgio Fedrizzi:** Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data.

## Data availability statement

Data will be made available on request.

## Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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