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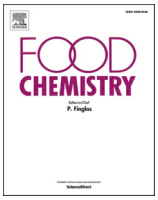

Cross-sectional, commercial testing, and chromatographic study of the occurrence of antibiotic residues throughout an artisanal raw milk cheese production chain

Gorka Santamarina-García [a,b,c,*], Gustavo Amores [a,b,c], Nagore Gandarias [d], Igor Hernández [a,b,c], Mailo Virto [a,b,c]

[a] Lactiker Research Group, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[b] Bioaraba Health Research Institute, Prevention, Promotion and Health Care Area, Lactiker-Nutrition and Health Line, Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[c] Joint Research Laboratory on Environmental Antibiotic Resistance, Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain
[d] Department of Biochemistry and Molecular Biology, Faculty of Pharmacy, University of the Basque Country (UPV/EHU), Paseo de la Universidad 7, 01006 Vitoria-Gasteiz, Spain

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ABSTRACT

This study investigated antibiotic utilization in artisanal dairies and residue occurrence throughout the raw milk cheese production chain using commercial testing (Charm KIS and Eclipse Farm^{3G}) and UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS. The cross-sectional survey results revealed gaps in the producers' knowledge of antibiotic use. Commercial testing detected antibiotic levels close to the LOD in 12.5 % of the samples, mainly in raw milk and whey, with 10.0 % testing positive, specifically in fresh and ripened cheeses, indicating that antibiotics are concentrated during cheese-making. Chromatographically, several antibiotics were identified in the faeces of healthy animals, with chlortetracycline (15.7 ± 34.5 μg/kg) and sulfamethazine (7.69 ± 16.5 μg/kg) predominating. However, only tylosin was identified in raw milk (3.28 ± 7.44 μg/kg) and whey (2.91 ± 6.55 μg/kg), and none were found in fresh or ripened cheeses. The discrepancy between commercial and analytical approaches is attributed to compounds or metabolites not covered chromatographically.

1. Introduction

Antibiotics are natural or synthetic compounds with antimicrobial activities that impact essential bacterial physiology and biochemistry, leading to either cell death (bactericide) or growth cessation (bacteriostatic) (Rossi et al., 2017; Virto et al., 2022). These compounds have been utilized for over 60 years to prevent and treat infections in livestock, such as mastitis. It is estimated that 73 % of global antibiotic use is dedicated to food-producing animals (Treiber & Beranek-Knauer, 2021). Antibiotics must be administered under veterinary prescription, employing authorized products and adhering to recommended doses, routes of administration, and withdrawal periods. Despite the fact that a significant portion of the product is eliminated through urine and faeces, residues can still be present in foodstuffs (Brocca & Salvatore, 2023; Virto et al., 2022).

Antibiotic residues are defined as pharmacologically active substances, including excipients or degradation products and their metabolites, that persist in food obtained from animals treated with the respective drugs (Brocca & Salvatore, 2023; Virto et al., 2022). The presence of antibiotic residues in food poses a public health threat due to the development of resistant bacteria and various toxicological effects, such as allergies, dysfunction of the intestinal microbiota, immunopathological effects, carcinogenicity, mutagenicity, nephropathy, hepatotoxicity, reproductive disorders, bone marrow toxicity, and anaphylactic shock (Virto et al., 2022). Consequently, regulatory authorities worldwide have established maximum residual limits (MRLs) based on acceptable daily intake (ADI), representing the amount of substance that can be ingested daily throughout life without appreciable

* Corresponding author.
E-mail addresses: gorka.santamarina@ehu.eus (G. Santamarina-García), gustavo.amores@ehu.eus (G. Amores), nagore.gandarias@ehu.eus (N. Gandarias), igor.hernandezo@ehu.eus (I. Hernández), mailo.virto@ehu.eus (M. Virto).

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health risks (Quintanilla et al., 2019a; Virto et al., 2022). Moreover, the United Nations pays special attention to the occurrence of antibiotic residues, classifying it as a factor contributing to the development of antimicrobial resistance. Consequently, it is an important target for the Sustainable Development Goals, particularly for goal 3, which aims to "ensure healthy lives and promote well-being for all at all ages" (WHO, 2017). Notably, antibiotic residues not only negatively impact health but also have environmental consequences (Virto et al., 2022). Soil and groundwater contamination have been reported due to the excretion of antibiotics through urine and faeces, as well as crop contamination through manure (Marshall & Levy, 2011; Virto et al., 2022). Farmers' lack of knowledge about antibiotic use and awareness of their potential impacts have been linked to poor practices, resulting in the presence of residues in both foodstuffs and the environment (Phares et al., 2020; Visschers et al., 2014). Therefore, concerted efforts from all stakeholders are necessary to ensure the proper use of antibiotics and prevent the spread of residues (Phares et al., 2020; Virto et al., 2022; Visschers et al., 2014).

Screening for the presence of antibiotic residues in food-producing animals is essential for ensuring food safety (Brocca & Salvatore, 2023). Different analytical methods have been developed worldwide to detect and quantify antibiotics. These methods include capillary electrophoresis, gas chromatography, and liquid or ultra-high-performance liquid chromatography (LC) (Sta Ana et al., 2021; Virto et al., 2022). Among these techniques, LC predominates because the analytes are polar, non-volatile, and thermally unstable compounds and do not require derivatization. LC can be coupled with different detectors, such as Ultraviolet (UV), Diode Array (DAD), Fluorescence Detector (FLD), mass spectrometer (MS), and tandem MS (MS/MS). However, a highly sensitive detector, such as MS/MS, is required since antibiotics are often found at low concentrations (Chiesa et al., 2020; Giraldo et al., 2022; Sta Ana et al., 2021; Virto et al., 2022). In addition, not all antibiotics absorb light; consequently, UV or FLD detectors are not appropriate without derivatization (Sta Ana et al., 2021). Ultra-high-performance liquid chromatography (UHPLC), also known as ultra-performance liquid chromatography (UPLC), represents a significant improvement owing to substantial enhancements in speed, resolution, and sensitivity (Wang, 2009). In addition to analytical methods, various qualitative techniques are available to food chain stakeholders for the rapid detection of antibiotic residues above legal limits in foodstuffs (Brocca & Salvatore, 2023; Chiesa et al., 2020; Virto et al., 2022; Wang, 2009), such as enzyme-linked immunosorbent assays (ELISA), surface plasmon resonance, biosensor technology, and microbial inhibition tests (Richards et al., 2022; Sullivan et al., 2022; Virto et al., 2022; Wang, 2009). Although microbial inhibition tests are easy to use, inexpensive, and have a high throughput, they lack specificity and sometimes show a high false-positive rate. Overall, screening tests based on microbial, enzymatic, or immunological receptor assays are faster and more selective than other methods (Wang, 2009).

Milk and dairy products are of great nutritional, social, and economic importance and are produced worldwide using various systems and technologies (Virto et al., 2022). Several studies have reported antibiotic residues in dairy products of animal origin (Rossi et al., 2017; Chiesa et al., 2020; Virto et al., 2022). However, MRLs have only been established for milk, and there are no limits for other dairy products, such as whey or cheese (Virto et al., 2022), in which these compounds can be concentrated (Quintanilla et al., 2019b). Moreover, antibiotic residues affect dairy production systems, such as the cheese-making process, due to problems with the growth of starter cultures, acidification, milk curdling, and ripening (Chiesa et al., 2020; Quintanilla et al., 2019b; Virto et al., 2022). To the best of our knowledge, there is no information in the literature on the prevalence of antibiotics along the entire dairy production chain because of livestock treatment, i.e., from animals to ripened cheese. The majority of published studies only analyse a single product, such as milk, or consist of intentionally spiked products to analyse the effect of different processes, such as sterilization (Cabizza et al., 2017; Giraldo et al., 2022; Quintanilla et al., 2019a; Quintanilla et al., 2019b).

Therefore, taking into account the need for information in this regard, this study aimed to achieve four primary objectives: (1) conduct a comprehensive survey on the knowledge, practices, and attitudes regarding antibiotic usage in artisanal dairies; (2) analyse the natural occurrence of antibiotic residues in healthy sheep herds through screening tests (Charm Kidney Inhibition Swab and Eclipse Farm^{3G} tests) and chromatographic techniques (UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS); (3) ascertain the extent to which antimicrobials may be transferred to raw milk; and (4) investigate the impact of the cheese-making process. This represents the first case study adopting a farm-to-fork strategy, examining the knowledge and practices of artisanal dairies, the natural presence of antibiotics in food-producing animals, and their dissemination throughout the entire dairy production chain.

2. Materials and methods

2.1. Area of study

To assess the current status of antibiotic use in small artisanal dairies and to determine the presence of antibiotic residues throughout the production chain of raw milk cheese, the European Protected Designation of Origin (PDO) Idiazabal cheese was selected for this study. The focus on Idiazabal PDO cheese stems from the fact that its production is primarily carried out by small family artisanal dairies. These dairies manage the entire process, from herd management to cheese-making and sales. Idiazabal cheese is a semi-hard or hard cheese produced from the raw milk of Latxa and/or Carranzana sheep breeds. The geographical area for livestock management and milk production suitable for cheese production is located in the Southern Basque Country, a region spanning 17,213.06 km^2 in southwestern Europe (43° 27′ − 41° 54′ N and 1° 5 ' − 3° 37′ W). This area corresponds to the natural habitats of diffusion of the sheep breeds. Flock management involves indoor forage and feed feeding in winter, with semi-extensive or extensive grazing in spring. Milk production, and the consequent elaboration of cheese primarily occurs between January and June, following the traditional seasonal method determined by the biological rhythms of the sheep (BOE, 1993).

2.2. Cross-sectional survey

A cross-sectional survey was conducted to evaluate the knowledge, practices, and attitudes of artisanal producers toward antibiotic use. A five-page questionnaire was designed based on previous studies reported in the literature (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020; Visschers et al., 2014). The questionnaire was validated for ethical suitability, comprehension, and technical aspects by the regulatory council of the Idiazabal PDO and a specialist veterinarian. The questionnaire, written in Spanish, was divided into sections covering sociodemographic characteristics of the producers and dairies (e.g., gender or education level) and knowledge and practices related to antibiotic utilization (such as the type of antibiotics used or frequency). Producers were informed about various aspects of the study, including objectives and data protection, during a PDO meeting. Surveys were conducted anonymously, and participants had the option to withdraw from the study at any time. Verbal consent was obtained from each participant before responding to the survey.

2.3. Sampling

Four producers, designated as A, B, C, and D, affiliated with the PDO, were chosen for sampling, each representing a specific geographical production area (Alava, Biscay, Gipuzkoa, or Navarre). The sampling period extended from March to July 2022, covering the annual production cycle. All producers adhered to uniform flock management and

cheese-making conditions in accordance with the specifications outlined by the Idiazabal PDO regulatory board (BOE, 1993). Each flock comprised approximately 350–400 Latxa breed sheep, managed from indoor feeding in winter to semi-extensive or extensive grazing in spring. Milking was automated, and milk was refrigerated (3–4 °C) until cheese-making. For cheese manufacturing, the milk was initially tempered to 25 °C, and the commercial mesophilic lyophilized starter culture Choozit MM 100 LYO 50 DCU (a mixture of *Lactococcus lactis* subsp. *lactis*, *Lactococcus lactis* subsp. *cremoris* and *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis*, DuPont NHIB Ibérica S.L., Barcelona, Spain) was used. Milk coagulation occurred at 28–32 °C for 20–45 min, employing artisanal rennet (extracted from the stomachs of Latxa or Carranzana lambs, obtained during the first month of lactation, cleaned, dried, salted, and ground) or commercial NATUREN® 195 Premium (Chr. Hansen Holding A/S, Hørsholm, Denmark). The cheeses were ripened in chambers maintained at 80–95 % relative humidity and temperatures of 8–14 °C for 60 days.

Samples, including faecal, raw milk, whey, fresh cheese, and 2-month-old ripened cheese, were collected from each producer. All samples were obtained from healthy flocks, excluding animals that had undergone veterinary treatment. Aseptic collection was performed in quadruplicate, with each set of samples corresponding to the same batch. Producers conducted the sampling, eliminating the need for approval from the Ethics Committee for Animal Experimentation. Informed verbal consent was obtained from dairies during samples collection. Samples were transported under refrigeration (3 ± 1 °C) to the laboratory and stored in a freezer (−80 ± 1 °C) for subsequent analysis. Cheese samples were defrosted at 5 ± 1 °C for 24 h and allowed to reach room temperature for 1 h before analysis.

2.4. Reagents and materials

Eclipse Farm^{3G} inhibition test was acquired from Zeulab S.L. (Zaragoza, Spain). Charm Sciences Inc. (Lawrence, Massachusetts, United States) supplied the Charm Kidney Inhibition (KIS) test and Feed Extraction Buffer (FEB). Chemicals including acetonitrile (≥99.9 %, LC-grade), methanol (≥99.9 %, LC- and UHPLC-grades), anhydrous citric acid (≥99.5 %), and sodium phosphate (≥98.0 %) were sourced from Scharlab (Barcelona, Spain). UHPLC-grade acetonitrile (≥99.9 %) was provided by Avantor Performance Materials (Gliwice, Silesia, Poland). Citrate buffer (≥99.0 %) was purchased from Honeywell Fluka (Charlotte, North Carolina, United States). Ultrapure water was obtained using a Milli-Q system (Millipore Corp., Billerica, Massachusetts, United States). Oasis HLB cartridges (60 mg, 3 mL; 200 mg, 6 mL; and 500 mg, 6 mL) were acquired from Waters Chromatography Division (Milli-Q water purification system, model 185, <0.05 μS/cm, Millipore, Bedford, Massachusetts, United States). Chemicals such as anhydrous sodium sulphate (≥99.0 %) and oxalic acid (≥99.9 %) were obtained from Merck (Darmstadt, Hesse, Germany). Sodium chloride (≥99.0 %), sodium hydroxide (reagent grade), and UHPLC-grade formic acid (≥98.0 %) were sourced from PanReac AppliChem (Castellar del Vallés, Catalonia, Spain). Clarify-PP Polypropylene filters (0.22 μm) were obtained from Phenomenex (Torrance, California, United States). LC-grade formic acid (≥98.0 %), trisodium citrate (≥99.0 %), polyvinylidene fluoride filter (0.45 μm) and antibiotics (analytical grade), namely ansamicines (rifaximin), β-lactams penicillins (amoxicillin, ampicillin, penicillin G, cloxacillin, dicloxacillin, nafcillin, and oxacillin), β-lactams cephalosporins (cephapirin + desacetylcephapirin, cefoperazone, cephalexin, cefquinome, cephalonium, ceftiofur + desfuroylceftiofur, cefazolin, cefacetril, and cefuroxime), lincosamides (lincomycin), macrolides (tylosin, erythromycin, spiramycin, and tilmicosin), sulfonamides (sulfadiazine, sulfathiazole, sulfapyridine, sulfamethazine, sulfameracin, sulfadimethoxine, sulfaquinoxaline, sulfamethizole, and sulfachloropyridazine), tetracyclines (tetracycline, oxytetracycline, chlortetracycline, and doxycycline), and quinolones (enrofloxacin + ciprofloxacin, danofloxacin, sarafloxacin, flumequine, marbofloxacin, and oxolinic acid) were purchased from Sigma-Aldrich (Madrid, Spain).

2.5. Screening analysis

Two commercially available tests were used for the screening of antibiotic residues throughout the Idiazabal raw milk cheese production chain: the Charm Kidney Inhibition Swab (KIS) test for faecal samples and the Eclipse Farm^{3G} test for dairy samples. Sample preparation and analysis were performed according to the manufacturer's instructions, with slight modifications, as described below.

2.5.1. Charm Kidney Inhibition Swab (KIS) test

In the preparation of faeces samples, 1 g of previously ground faeces was combined with 30 mL of FEB and allowed to sit for 5 min. The mixture was then vigorously shaken for 1 min, followed by a 1-min settling period for the solids before conducting the test. Subsequently, the Charm Kidney Inhibition Swab (KIS) test swab was immersed in the stool extract for 10 s to facilitate absorption.

The Charm KIS test was executed following the manufacturer's instructions. This test relies on *Geobacillus stearothermophilus* cultured on agar with a purple pH indicator. In the absence of antibiotics, the bacteria proliferated during incubation, producing an acid that changed the medium's colour to yellow. Conversely, in the presence of antibiotics above the limit of detection (LOD), growth was inhibited, and the medium retained its blue/purple colour. Consequently, the swab containing the sample extract was introduced into the device and incubated at 64 ± 2 °C (Biometra TB1 Thermoblock, Gottingen, Germany) for the time required for a known antibiotic-negative sample to transition to yellow (approximately 3 h, depending on the lot). Results were interpreted through visual assessment of the colour change in the culture medium after incubation. Samples were categorized as positive (blue or purple) or negative (yellow). The LODs of the Charm KIS test are detailed in Supplementary Table 1

2.5.2. Eclipse Farm^{3G} test

The dairy samples underwent preparation and analysis in accordance with the manufacturer's instructions, with minor adjustments. In the case of raw milk samples, a 100 μL aliquot was dispensed into an Eclipse Farm^{3G} test tube using disposable pipettes supplied with the kit. The tubes were subsequently sealed and allowed to diffuse for 1 h at room temperature. Following incubation, 2–3 washes with deionised water was conducted, and the tube surfaces were dried and resealed with adhesive foil. The procedure for whey samples mirrored that of raw milk, with the caveat that the pH of the whey was maintained within the range of 6.5–7.0 (Diserens, 2014). For cheese samples, preparation followed the guidelines outlined in Bulletin N° 471/2014 of the International Dairy Federation (Diserens, 2014). In summary, 30 g of cheese was combined with 70 mL of antibiotic-free milk, pre-warmed to 45 °C, and homogenized for 4 min in a stomacher (Masticator Basic 400; IUL Instruments, Königswinter, Germany). The resulting suspension underwent centrifugation at 3800 × g for 10 min, and the aqueous extract was retained. The pH was adjusted to 6.5–7.0 with 2 N sodium hydroxide. A 100 μL aliquot of this solution underwent processing in the same manner as explained earlier for milk and whey samples.

The Eclipse Farm^{3G} test was executed according to the manufacturer's specifications for the analysis of sheep milk. The Eclipse Farm^{3G} is designed to identify more than 50 antibiotics, encompassing beta-lactams, tetracyclines, sulfonamides, macrolides, lincosamides, and ansamycins. This assay relies on inhibiting microbial growth. The Eclipse Farm^{3G} tubes were equipped with a specific culture medium containing *Geobacillus stearothermophilus* spores and a pH indicator. In the absence of antibiotics (negative), incubation led to spore germination and multiplication, resulting in the production of acidic compounds that reduced the pH and changed the colour of the medium. Conversely, if the sample contained an antibiotic concentration surpassing the limit of detection (positive), microbial growth was hindered. Subsequently,

the tubes, following 2–3 washes with deionized water post-incubation at room temperature for 1 h, underwent further incubation at 65 ± 1 °C (Biometra TB1 Thermoblock, Gottingen, Germany). The duration of incubation was the time required for a known antibiotic-negative sample to transition to yellow (ranging from 2 h 15 min to 3 h, depending on lot and sample type). Test results were interpreted through visual assessment of the colour change in the medium after incubation, classifying samples as positive (blue-purple), negative (yellow), or close to the LOD (green–blue, indicating the presence of antibiotics at a concentration near the LOD). The LODs for the Eclipse Farm^{3G} test are provided in Supplementary Table 2

2.6. Chromatographic analysis of antibiotic residues

2.6.1. Solid-phase extraction (SPE) coupled to ultra-high-performance liquid chromatography-triple quadrupole mass spectrometry (UHPLC-QqQ-MS/MS)

The extraction and analysis of antibiotic residues in the faecal samples followed established and validated protocols at the Department of Analytical Chemistry and the Research Centre for Experimental Marine Biology and Biotechnology (PIE) of the University of the Basque Country (Vergara-Luis et al., 2023). Briefly, 2 g of homogenized faeces samples underwent extraction with 760 μL of Milli-Q water, 5 mL of acetonitrile, 2 g of anhydrous sodium sulphate, 0.5 g sodium chloride, 0.25 g anhydrous citric acid, and 0.025 g sodium phosphate. A ceramic homogeniser was added to the mixture, manually shaken and degasified until no gas was released. Samples were vortexed at 500 × g for 8 min at 10–15 °C, centrifuged at 2100 × g for 5 min at 10–15 °C, and 2 mL of the extract were diluted to 40 mL with 0.05 M citrate buffer (pH 4). Subsequently, using Solid-Phase Extraction (SPE), the diluted extract was loaded onto a 500 mg Oasis HLB cartridge, previously conditioned with 10 mL of acetonitrile, 10 mL of Milli-Q water, and 10 mL of citrate buffer. Cartridges were washed with 5 mL water, dried under vacuum, and eluted with acetonitrile (9 mL). The extracts were evaporated to 1 mL under a nitrogen flow (TurboVap LV evaporator, aliper Life Sciences, Hopkinton, MA, United States), and 125 μL of the resulting extract were reconstituted in 250 μL of 50:50 (v:v) 0.01 M acetonitrile:oxalic acid (pH 2) and filtered through 0.22 μm polypropylene filters.

Antibiotic residues were analysed using an Agilent 1290 Infinity II UHPLC system (Agilent Technologies, Madrid, Spain) equipped with a degassing system, binary pump, and automatic injector, coupled to an Agilent 6430 Triple Quad tandem mass-spectrometer (QqQ) (Agilent Technologies), as previously described (Vergara-Luis et al., 2023). Antibiotic compounds were separated on a Kinetex C18 polar column (100 Å pore size, 2.6 μm particle size, 2.1 mm i.d. × 50 mm length) (Phenomenex, Alcobendas, Spain). Both column and pre-column (Kinetex C18 polar, 100 Å pore size, 2.6 μm particle size, 2.1 mm i.d. × 5 mm length) were maintained at 35 °C, and two solvents were used: (A) 0.1 % (v/v) UHPLC quality water in formic acid and (B) 0.1 % (v/v) UHPLC quality methanol in formic acid. The injection volume was fixed at 3 μL, the temperature at 35 °C, and the flow rate was established at 0.3 mL/min.

The chromatographic data obtained were analysed using the Agilent MassHunter Workstation software (Quantitative Analysis for QqQ, 10.0 version, Agilent Technologies). Antibiotic compounds were identified by MS/MS detector, and quantification was performed in dynamic multiple reaction monitoring (DMRM) acquisition mode, using nitrogen (99.999 %, Air Liquide, Paris, Île-de-France, France) as nebulizer and drying and collision gas (99.999 %, Messer, Bad Soden am Taunus, Hessen, Germany). The electrospray ionization source operated in positive ion mode (ESI +) for all analytes. The gas temperature was maintained at 300 °C, with a drying flow rate of 8 L/min, the capillary voltage was set at 3 kV, and the nebulizer pressure at 50 psi. Parameters related to mass spectrometry (fragmentor voltage, collision energy, or collision cell accelerator) were optimised by a standard containing all target compounds at a concentration level of 2.5 μg/mL through the specific Agilent MassHunter Optimizer software (10.0 version), considering both target analytes and surrogates.

2.6.2. Solid-phase extraction (SPE) coupled to liquid chromatography-triple quadrupole mass spectrometry (LC-QqQ-MS/MS)

The extraction and analysis of antibiotic compounds in dairy samples were conducted in accordance with established and validated protocols at the Instituto Lactológico de Lekunberri (Lekunberri, Navarre), as described by Quintanilla et al. (2019b), with slight modifications. In summary, 10 g or mL, depending on the sample type, were combined with 20 g trisodium citrate (20 % wt./wt.) and homogenized for 3 min at 40 °C twice in a stomacher. The resulting mixture underwent centrifugation at 9000 × g for 10 min at room temperature, and 2 g of the supernatant were extracted by SPE using an Oasis HLB cartridge preconditioned with 1 mL of methanol and 1 mL of ultrapure water. The cartridge was rinsed with 2 mL water, eluted with 2 mL methanol, and dried under vacuum. Subsequently, 500 μL of formic acid was added and homogenized for 5 min in an ultrasonic bath. The resulting extracts were filtered using 0.45 μm polyvinylidene fluoride filters.

Antibiotics were analysed using an Alliance 2695 Liquid Chromatography system equipped with a diode-array detector and a Micromass Quattro MicroTM triple quadrupole tandem mass spectrometer (QqQ) (Waters Chromatography Division), as previously described with minor modifications (Quintanilla et al., 2019b). Antibiotics were separated on an XBridge C18 column (100 mm length, 34.6 mm, 2.1 mm i.d., 3.5 μm particle size, Waters Chromatography Division) using two solvents: (A) 0.1 % (v/v) formic acid in water and (B) 0.1 % (v/v) formic acid in acetonitrile. The gradient program was as follows: 0–8 min, 95 % A and 5 % B; 8–14 min, 25 % A and 75 % B; 14–15 min, 5 % A and 95 % B; and 15–20 min, 95 % A and 5 % B. For oxytetracycline, the gradient program consisted of: 0–6 min, 85 % A and 15 % B; 6–8 min, 82 % A and 18 % B; 8–15 min, 50 % A and 50 % B; and 15–20 min, 85 % A and 15 % B. The injection volume was established at 20 μL, and the flow rate was set at 0.2 mL/min.

The chromatographic data obtained were analysed using Mass-Lynx software (version 4.0; Waters Chromatography Division). Antibiotics were identified using an MS/MS detector and electrospray ionization in positive ion mode (ESI +). The source temperature was maintained at 140 °C, the needle voltage was set at 3.0 kV, lens voltage at 0.2 V, desolvation and cone gas (nitrogen) flow were set at 750 and 50 L/h, respectively; and desolvation temperature was maintained at 450 °C. Typical recoveries were approximately 85 to 100 % for the β-lactams and tetracyclines, 80 to 95 % for the macrolides, and 90 to 110 % for the quinolones. Calibration curves were generated for each antibiotic.

2.7. Food safety margin (FSM) calculation

To assess the risk to consumer health arising from the consumption of dairy products (milk, whey, or cheese) containing antibiotic residues, the Food Safety Margin (FSM) indicator was employed. The FSM value is calculated, as proposed by Quintanilla et al. (2019a), as follows:

$$FSM = \begin{cases} 0 & if\ \ HQ_i > 1 \\ 1 - HQ_i & if\ \ HQ_i \leq 1 \end{cases}$$

where

$$HQ_1 = \frac{(H_i \times C)/W}{ADI_i}$$

Where H_i is the concentration of the detected antibiotic (μg/kg), C corresponds to the daily intake (kg/person/day), W represents the mean body weight (kg) according to the age, and ADI_i is the acceptable daily intake. The numerator corresponds to the calculation of the estimated daily intake (EDI_i).

2.8. Statistical analysis

The data treatment and analysis involved the utilization of various packages and software. Data preparation and analysis were conducted utilizing IBM SPSS statistical package version 28.0 (IBM SPSS Inc., Chicago, IL, United States, 2019). Descriptive statistics, encompassing means and standard deviations, were employed for data summarization. Survey data analysis employed Pearson's chi-squared test to analyse the association between variables (dairies and producers' sociodemographic characteristics, practices, and knowledge related to antibiotic administration) at a 95 % confidence interval. The degree of association was assessed using Cramer's V test. Kruskal–Wallis one-way analysis of variance (ANOVA) with Bonferroni correction was executed using the SPSS package to analyse the significance ($P \leq 0.05$) of variations in screening test results and antibiotic concentrations (n.d. values were substituted with zeros) among samples based on the production chain and producer factors. Permutational multivariate analysis of variance (PERMANOVA) was carried out in RStudio version 1.4.1717 and R version 4.1.1 (R Core Team, Vienna, Austria, 2021) with the "vegan" package (https://github.com/vegandevs/vegan) to investigate the overall impact of production chain and producer factors on screening test results and antibiotic concentrations. Hierarchical Clustering Analysis (HCA) was employed to analyse groupings and trends of screening test results according to the production chain and producer factors. The analysis was performed with Unit Variance (UV) scaled data and presented in a heat-map using the "gplots" package (https://github.com/cran/gplots) in R. Principal Component Analysis (PCA) was conducted using SIMCA software version 17.0.2.34594 (Umetrics AB, Umeå, Sweden) to assess antibiotic concentration trends based on the production chain and producer factors. The number of principal components (PC) was determined through eigenvalues and cross validations. Orthogonal Partial Least Squares Discriminant Analysis (OPLS-DA) was also executed in SIMCA to investigate potential differences in antibiotic concentrations among samples based on production chain and producer factors. Production chain and producer factors were utilized as Y-variables, while antibiotic concentrations served as X-variables. Model validation involved R2 and Q2 values, permutation tests, and Inner Relation plots. The significance of each antibiotic in the model was evaluated using Variable Influence on Projection (VIP) values and loading weights.

3. Results and discussion

3.1. Cross-sectional survey

3.1.1. Sociodemographic characteristics of dairies

The cross-sectional surveys or questionnaires employed in this study represent the most widely utilized methods for gathering information on antibiotic utilization or knowledge (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020). Supplementary Table 3 presents the sociodemographic characteristics of the Idiazabal PDO dairies participating in this investigation. Overall, a well-balanced distribution in terms of producers' gender was observed, with a slight predominance of females (53.3 %). All producers had received education, primarily through vocational training (66.7 %). However, the majority of their studies were unrelated to their professional activities (66.7 %), and all indicated participation in courses. A large proportion of producers had Internet access (86.7 %) and occasionally referred to articles on livestock production and health (76.9 %). Professional experience exceeding 10 years was common among producers (60 %), and 86.7 % indicated membership in livestock associations. The majority of dairies were situated outside urban cores (73.3 %), with an average annual production of 47,000 litters of raw ewe milk and 3,744 kg of cheese. Concerning flock management, producers reported an average barn confinement of 3.04 $\pm$ 0.782 months, with an average barn area of 643 $\pm$ 155 m^2. Grazing primarily occurred around the dairy (66.7 %), with an area exceeding 5 ha in most cases (86.7 %). Additionally, 66.7 % of producers mentioned grazing in mountainous areas. Mechanical milking systems predominated (86.7 %), and all producers exclusively utilized milk from their own herds for cheese production. To the best of our knowledge, there is a paucity of survey studies on antibiotic utilization in dairy sheep (Lee et al., 2022). Published studies on dairy cattle primarily concentrate on cattle (Casseri et al., 2022), while among food-producing animals, pigs and poultry predominate (Bedekelabou et al., 2022). Overall, the sociodemographic characteristics of dairy farms exhibit notable differences based on cattle type and geographical location (Casseri et al., 2022; Dyar et al., 2020; Phares et al., 2020).

3.1.2. Antibiotics utilization and knowledge of producers

Table 1 presents participants' knowledge and attitudes regarding antibiotic use. The majority of producers lacked formal or informal training in antibiotics, although 60.0 % were able to accurately define antibiotics. Concerning antibiotic effectiveness, a significant proportion expressed uncertainty about whether antibiotics were currently less effective than in the past (60.0 %), although discrepancies were observed. Nevertheless, the majority expressed concerns regarding antibiotic resistance (93.3 %). The lack of knowledge about antibiotics and their use, coupled with disparities in current use, effectiveness, and concerns about antimicrobial resistance, aligns with findings from other published studies (Casseri et al., 2022; Lee et al., 2022; Moreno, 2014; Phares et al., 2020; Visschers et al., 2014).

Of the producers, 93.3 % indicated having administered antibiotics to their flock at least once, usually less than once a month, in the previous year (64.3 %). The antibiotics administered included β-lactams, such as penicillin G or amoxicillin, and tetracyclines, such as tetracycline and oxytetracycline. The use of antibiotics in farms is a widespread practice (Bedekelabou et al., 2022; Lee et al., 2022; Phares et al., 2020). Penicillin and tetracycline are the most commonly used veterinary antibiotics (Casseri et al., 2022; Dyar et al., 2020), although the compounds and doses vary between farms and territories (Bedekelabou et al., 2022; Casseri et al., 2022; Visschers et al., 2014). A significant portion of producers (53.3 %) mentioned the ease of obtaining antibiotics, always through veterinary services, unlike other studies where obtaining antibiotics without a prescription has been observed (Dyar et al., 2020; Lee et al., 2022; Phares et al., 2020). Most producers reported using veterinary services for treating livestock diseases once or twice a year (46.7 %), with fewer indicating more than twice a year (40.0 %). The use of veterinary services varies significantly by territory, with developing countries having the least use (Bedekelabou et al., 2022; Dyar et al., 2020; Phares et al., 2020). Lee et al. (2022) reported the effects of farm size, with small producers having the highest utilization. Visschers et al. (2014) suggested that the use of these services promotes the correct use of antibiotics. Veterinarians were responsible for administering antibiotics in only 13.3 % of cases, with the majority administered by the dairy owner or some employees (35.7 % and 28.6 %, respectively). The 92.9 % of the producers indicated using antibiotics only when they were certain about a bacterial infection, whereas the 7.14 % indicated also giving antibiotics when the animal appears to be sick. Additionally, 14.3 % used antibiotics for prevention, apart from curative purposes. In all cases, the recommended dose was reported to be respected, and the route of administration was parenteral. Farm workers or owners administering antibiotics, along with improper use and incorrect concentrations, have been reported in other studies (Bedekelabou et al., 2022; Dyar et al., 2020; Moreno, 2014; Phares et al., 2020), emphasizing that producer education is a crucial factor (Bedekelabou et al., 2022). Bedekelabou et al. (2022) have reported that producers in Togo mainly used antibiotics for preventive purposes, whereas Moreno (2014) indicated that Spanish producers did not clearly distinguish between curative and preventive purposes. In this study, antibiotics were not administered through feed and water, unlike in other studies (Dyar et al., 2020; Lee et al., 2022), which has been associated with antibiotic overuse and the spread of antimicrobial

Table 1
Antibiotics utilization and knowledge of producers.

Item	Percentage (%)
Training (formal or informal) on antibiotics	
Yes	6.67
No	86.7
No answer/don't know	6.67
Correct description of what antibiotics are	
Yes	53.3
No	40.0
No answer/do not know	6.67
In your experience, antibiotics are now less effective than in the past:	
I strongly disagree	0.00
I do not agree	13.3
Not sure	66.7
I agree	0.00
I strongly agree	6.67
No answer/don't know	13.3
Are you worried about antibiotic resistance?	
Yes	93.3
No	0.00
I do not know what antibiotic resistance is	0.00
No answer/don't know	6.67
Ever given antibiotics to animals	
Yes	93.3
No	6.7
Ease or difficulty of obtaining antibiotics	
Easy	53.3
Difficult	33.3
According to the veterinary staff	6.7
No answer/do not know	6.7
Antibiotics used	
Amoxicillin	5.26
Penicillin G	36.8
Dihydrostreptomycin	26.3
Oxytetracycline	15.8
Polymyxin b	10.5
Tetracycline	5.3
Antibiotics adquisition	
Veterinary staff	80.0
Veterinary staff of external company	13.3
Association	6.7
Availability of veterinary staff	
Always	33.3
Sometimes	53.3
Never	0.0
No answer/do not know	13.3
Veterinary services's use for the treatment of livestock diseases	
Never	6.67
Once a month	13.3
1 or 2 times a year	46.7
More than 2 times a year	40.0
Person administering antibiotics	
Veterinary staff	14.3
Employee	28.6
Owner	35.7
Veterinary staff and/or owner	21.4
Administered dose	
Dosage recommended by the veterinarian	100
Dose given based on your experience	0.0
Dose administered based on the recommendation of another farmer	0.0
When antibiotics are given to animal?	
When I am sure that the animal has a bacterial infection	92.9
When I am sure that the animal has a bacterial infection, whenever the animal is sick and/or whenever the animal appears ill	7.14
Why are antibiotics used?	
Prevent and cure (treat infections)	14.3
Prevent	0.0
Cure (treat infections)	85.7
Frequency of antibiotic administration during the last 12 months	
More than once a week	0.0
Once a week	0.0
More than once a month	7.14
Once a month	0.0
Less than once a month	64.3
According to the need	21.4
No answer/do not know	7.14
Method of antibiotics administration	
Along with the feed	0.0
Along with the drinking water	0.0
Parenterally (injected)	100
Herd management during antibiotic administration	
Antibiotics are given always or often along with feed or drinking water to keep animals healthy and prevent disease.	0.0
Antibiotics are given to all animals in the herd, when some of the animals are sick.	0.0
Antibiotics are given only to sick animals	100
How long does it take for the antibiotic to disappear from the milk?	
Same day of treatment	0.00
3 days after treatment	0.00
One week after treatment	0.00
According to the antibiotic	92.9
I do not know.	7.14
Has milk or cheese ever been tested for the presence and/or concentration of antibiotics?	
Yes, routine within dairy control	53.3
Yes, once within dairy control	6.67
Yes, once within dairy control and sometime of my own free will	6.67
Yes, routine within dairy control and sometime of my own free will	6.67
Never	26.7
Method used to examine the presence and/or concentration of antibiotics in milk or cheese	
Inhibitor analysis	13.3
No answer/don't know	86.7

resistance (Treiber & Beranek-Knauer, 2021). The 92.9 % of the producers indicated that the withdrawal period of an antibiotic depended on the compound. However, only 53.3 % of producers routinely examined the presence and concentration of antibiotics in milk and cheese, with some doing so voluntarily. Literature addressing adherence to withdrawal times and the analysis of residues is limited (Phares et al., 2020). Ensuring compliance with the withdrawal period is crucial to guarantee the absence of the compound in both animal organisms and derived products, such as milk (Virto et al., 2022).

Regarding the association between the sociodemographic characteristics of the dairies and producers and the practices and knowledge related to antibiotic use, it was observed that producers with lower education levels (primary education) were the only ones with training in relation to antibiotics ($P < 0.01$). Moreover, it was observed that those producers who used antibiotics to cure and prevent infections were only those whose training was related to the professional field ($P < 0.05$), indicating a lack of knowledge on antibiotic utilization (Moreno, 2014). Overall, fewer associations were observed compared with other studies (Dyar et al., 2020; Lee et al., 2022; Phares et al., 2020). For instance, Bedekelabou et al. (2022) have observed that knowledge about antibiotics differs according to the producer's gender, and producers with lower education are more likely to misuse antibiotics. Phares et al. (2020) found an association between farm location, income status, and antibiotic use in Ghana.

Notably, several associations were observed between the producers' knowledge and practices regarding antibiotic use. The results revealed that producers who received training on antibiotics analysed the presence of residues ($P < 0.01$). A large number of producers used antibiotics only when they were sure that the animal had a bacterial infection and routinely analysed the antibiotic residues ($P < 0.01$). The use of antibiotics and the reasons for their use were also significantly associated ($P < 0.05$). Most producers who used antibiotics when they were sure that the animal had a bacterial infection only used it to cure the animal, whereas those producers who administered antibiotics without being sure that it was a bacterial infection used them to prevent infection. A

relationship was also observed between the administration of antibiotics and opinions on their effectiveness ($P < 0.01$). Producers who had never administered antibiotics indicated that antibiotics were less effective today than in the past, whereas most producers who administered antibiotics indicated that they were not sure and, to a lesser extent, disagreed with the current lower effectiveness. In addition, the frequency of antibiotic use was associated with opinions on the effectiveness of antibiotics ($P < 0.05$). Most producers who were unsure of the current effectiveness of antibiotics had administered antibiotics to the flock less than once per month, while those who did not agree that antibiotics are less effective today indicated that there was no frequency of annual or monthly use since it corresponds to the need. Overall, these associations confirm that the formation of producers in terms of antibiotic knowledge and practices is an important factor in ensuring the correct utilization of these compounds and avoiding their presence in the food production chain (Bedekelabou et al., 2022; Dyar et al., 2020).

3.2. Antibiotics residues determination throughout cheese production chain by screening tests

Fig. 1 depicts the outcomes of the Charm KIS and Eclipse Farm^{3G} antibiotic screening tests. A noteworthy 76.3 % of the samples collected across the production chain yielded negative results, whereas 12.5 % (10/80) approached the LOD, and 10.0 % (8/80) yielded positive results (Fig. 1A). Although differences among producers were observed, they did not attain statistical significance ($P > 0.05$) (Fig. 1A). Across the production chain, statistically significant differences among sample types (faeces, raw milk, whey, fresh cheese, and ripened cheese) were observed ($P \leq 0.01$) (Fig. 1B-C). Employing a multivariate approach, PERMANOVA confirmed the impact of sample type ($P \leq 0.01$) and the non-significant impact of the producer factor ($P > 0.05$). Differentiation of samples based on antibiotic screening test results was further confirmed using HCA (Fig. 1A and 1D).

All faecal samples, irrespective of the producer ($P > 0.05$), tested negative in the Charm KIS test (Fig. 1), indicating the absence of antibiotic residues above the LOD. These findings are particularly

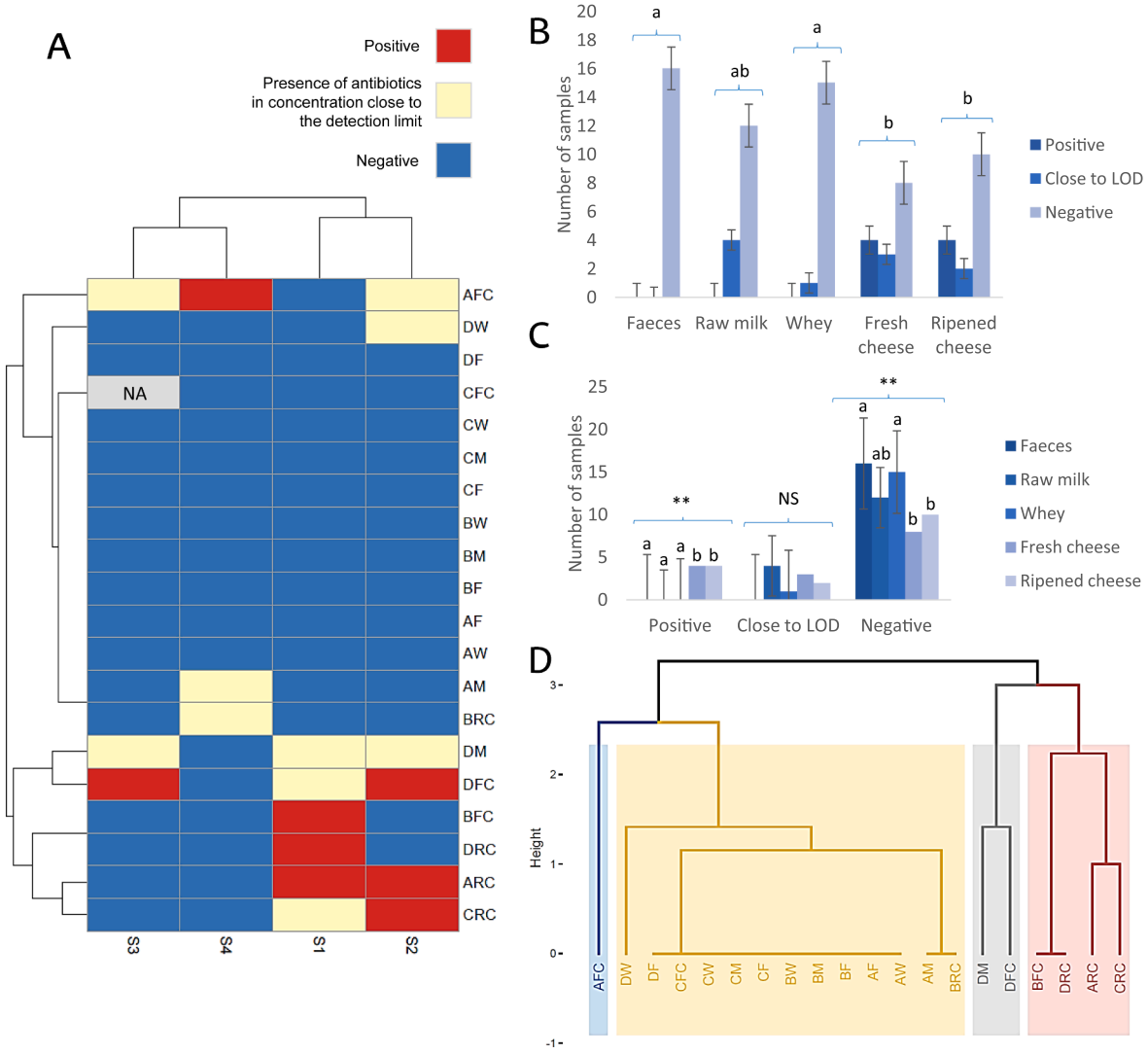

Fig. 1. HCA heat map (A), bar plots (B and C) and HCA dendogram based on the screening test (Charm KIS and Eclipse Farm^{3G}) results. The different letters within the group of bars indicate a significant difference at $P \leq 0.01$. Abbreviations: NA: not possible to analyse; S1: first sampling week; S2: second sampling week; S3: third sampling week; S4: fourth sampling week; AF, BF, CF, DF: faeces samples from producers A, B, C and D, respectively; AM, BM, CM, DM: raw milk samples from producers A, B, C and D, respectively; AW, BW, CW, DW: whey samples from producers A, B, C and D, respectively; AFC, BFC, CFC, DFC: fresh cheese samples from producers A, B, C and D, respectively; ARC, BRC, CRC, DRC: ripened cheese samples from producers A, B, C and D, respectively; **: $P \leq 0.01$; NS: non-significant, $P > 0.05$.

noteworthy given reports from various studies highlighting the potential transfer of manure antibiotics to the environment, with implications for soil microbiota, groundwater quality (Virto et al., 2022), and crops, posing a risk to human health (Zhang et al., 2022). However, no screening tests for faecal samples have been reported to date.

Similarly, the Eclipse Farm^{3G} test revealed that none of the raw milk samples collected tested positive for antibiotics (Fig. 1). Nevertheless, 25.0 % (4/16) of the samples exhibited concentrations close to the LOD, with no significant variation according to the producer ($P > 0.05$). The presence of residues in raw milk as opposed to animal faeces is meaningless (Fig. 1), since the administered compounds are primarily excreted through urine and faeces (Virto et al., 2022). The observed incongruity may be attributed to variations in the LODs of each test. Notably, the Charm KIS test features LODs 10^2–10^3 times higher than those of the Eclipse Farm^{3G} (Supplementary Tables 1 and 3). Consequently, higher concentrations are necessary for a positive result. For instance, a positive result for oxytetracycline in faeces would require 10^5 μg/L, while only 150 μg/L would be needed for milk. Based on the established MRLs, all raw milk samples can be classified as compliant (Brocca & Salvatore, 2023). These results agree with the latest results reported by the European Food Safety Authority (EFSA) for the monitoring of antibiotics in milk, which reported only 0.23 % (47/20407) of non-compliant samples, maintaining the low rate trend observed in the last decade (0.09–0.44 %) (Brocca & Salvatore, 2023). The Food and Drug Administration Agency of the United States (FDA) has also reported a low percentage of non-compliant raw milk samples in the United States over the last year (0.009 %) and the past decade (0.02–0.008 %) (FDA, 2023). However, these studies primarily focus on bovine milk. Although several studies have addressed the detection of antibiotic residues in raw milk through screening tests, most pertain to bovine milk, and information on milk obtained from small ruminants such as sheep is limited (Virto et al., 2022). Yamaki et al. (2006) identified a 2.6 % positivity rate in raw milk samples from the Assaf breed, while Gonzalo et al. (2010) reported a 0.6 % rate for raw milk of the Manchega breed. These rates imply higher noncompliance compared to the findings of this study.

Throughout the cheese-making process, none of the collected whey samples yielded a positive result, regardless of the producer ($P > 0.05$), with 6.25 % (1/16) of the samples yielding a result close to the LOD and predominating in a negative result (93.8 %) (Fig. 1). Concerning the fresh cheese samples, 18.8 % (3/16) yielded results close to the LOD, and 25.0 % (4/16) yielded a positive result. Nevertheless, some false positives or false close to the LOD results for fresh cheeses were observed, because the milk samples used to make the cheeses yielded negative results, which constituted 3.75 % (3/80) of the total samples. False-positive results for screening tests have been linked to substances that inhibit bacterial growth, which in this case could be related to lysozyme or free fatty acid content (Richards et al., 2022). However, this false-result rate cannot be compared with the literature because no results have been published on antibiotic screening tests in cheese, substantiating the novelty of these results. It is essential to improve the sensitivity and precision of inhibition tests to ensure food safety and avoid economic losses to producers due to the elimination of samples incorrectly classified as containing antibiotics and the unnecessary performance of confirmatory analytical tests (Chiesa et al., 2020). Without considering false results, 20.0 % (3/16) of the fresh cheese samples were positive, and 6.67 % (1/16) gave results close to the LOD. Few studies have analysed the effect of the cheese-making process on antibiotic concentrations; however, the retention of antibiotics in curd or elimination by whey during the cheese-making process depends on the compound and its characteristics (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b; Virto et al., 2022). These results indicate that the Idiazabal cheese-making process leads to a change in the screening test results from close to the LOD in milk to positive in fresh cheese; consequently, the antibiotics present in milk could be transferred and concentrated, mainly in the cheese. Notwithstanding the lack of MRLs for whey or cheese, as these products are transformed from raw milk and considering the MRLs, all whey samples were classified as compliant, whereas 20.0 % (3/16) of the fresh cheeses were possibly non-compliant (Diserens, 2014).

During ripening, a similar trend was observed, with 25.0 % (4/16) of the ripened cheese samples yielding a positive result and 12.5 % (2/16) being close to the LOD. However, false positives or false close to the LOD accounted for 6.25 % (5/80) of the total samples, which could be related to higher concentrations of these compounds inhibiting bacterial growth (Richards et al., 2022; Santamarina-García et al., 2022), as mentioned earlier. Thus, aside from false results, no ripened cheese close to the LOD was observed, and 6.25 % (1/16) were positive, and consequently, possibly non-compliant (Diserens, 2014). It is noteworthy that 100 % (3/3) of the possibly non-compliant fresh cheeses became compliant after ripening, confirming the degradative effect of the ripening process, although it has been little studied (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b; Virto et al., 2022). However, concerning the unique positive ripened cheese sample observed, its corresponding fresh cheese sample yielded a result close to the LOD (Fig. 1), suggesting a concentration effect during ripening, which has been little studied so far (Quintanilla et al., 2019b). Considering that national and international food safety authorities, such as the EFSA and FDA, do not monitor the presence of antibiotic residues in dairy products (Brocca & Salvatore, 2023; FDA, 2023), these results are of special interest because they indicate that, in most cases, the ripening process reduces the risk to consumers' health (Virto et al., 2022). Nonetheless, in some cases, residues could be maintained up to the final cheese; therefore, more research should be conducted to identify cheese-making or ripening conditions that could concentrate residues.

3.3. *UHPLC-QqQ-MS/MS and LC-QqQ-MS/MS analysis of antibiotic residues' occurrence throughout cheese production chain*

Different national and international regulations stipulate that samples yielding non-compliant results through screening tests must undergo analysis using confirmatory methods to identify and quantify antibiotic compounds (Brocca & Salvatore, 2023; Diserens, 2014). Consequently, the concordance between screening tests and chromatographic analysis was assessed. Table 2 presents the average concentrations of antibiotic compounds identified throughout the Idiazabal cheese production chain. The results revealed that 26.3 % (21/80) of the samples were above the LOQ. However, only 13.0 % of the tested antibiotics were identified, specifically a macrolide (tylosin), a quinolone (enrofloxacin), two sulfonamides (sulfadiazine and sulfamethazine), and two tetracyclines (oxytetracycline and chlortetracycline) (Table 2). Notably, chlortetracycline was the most abundant compound, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg). It is worth mentioning that, in the cross-sectional survey (Table 1), only tetracyclines were reported among these compounds, possibly indicating a lack of producers' awareness of the provided antibiotics (section 3.1). Ansamicine, β-lactam (penicillin and cephalosporin), or lincosamide class antibiotics were not detected, despite being mentioned in the survey (Table 1), suggesting potential lack of recent use. Overall, these results affirm the efficacy and precision of LC coupled with MS/MS for detecting and quantifying antibiotics at low concentrations (Sta Ana et al., 2021).

According to the detected compounds, tetracyclines are natural broad-spectrum compounds produced by *Streptomyces aureofaciens* and *Streptomyces rimosus*, acting against gram-positive and gram-negative bacteria such as *Chlamydia*, *Mycoplasma*, *Rickettsiae*, and protozoan parasites (Alcock et al., 2023). However, recent years have seen high resistance rates reported in various bacteria (Virto et al., 2022). The European Medicines Association (EMA) categorizes these compounds as category D, designating them as first-line treatments and recommending cautious use only when medically necessary (CVMP and CHMP, 2020). The detected macrolide, tylosin, is also a natural compound produced by

Table 2
Concentration (mean ± standard deviation) of the antibiotic compounds identified throughout production chain of Idiazabal cheese (faeces, raw milk, whey, fresh cheese and ripened cheese) in four producers (A, B, C and D).

Antibiotics Class	Compound	Antibiotic concentration (μg/kg)[1]																				P-value[2]	
		Faeces Producer				Raw milk Producer				Whey Producer				Fresh cheese Producer				Ripened cheese Producer				C	P
		A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D	A	B	C	D		
Ansamycin	Rifaximin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (penicillin)	Amoxicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ampicillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Penicillin G	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Dicloxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Nafcillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxacillin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
β-Lactam (cephalosporin)	Cephapirin + Desacetylcephapirin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefoperazone	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalexin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefquinome	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cephalonium	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Ceftiofur + Desfuroylceftiofur	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefazolin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefacetril	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Cefuroxime	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Lincosamide	Lincomycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Macrolide	Tylosin	n.d.	n.d.	n.d.	n.d.	**6.15 ± 12.3**	n.d.	**6.98 ± 8.24**	n.d.	**5.35 ± 10.7**	n.d.	**6.28 ± 7.39**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.090	0.052
	Erythromycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Spiramycin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Tilmicosin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Quinolone	Enrofloxacin + Ciprofloxacin	n.d.	**2.05 ± 2.90**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	0.092	0.392
	Flumequine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sarafloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000

(*continued on next page*)

Table 2 (*continued*)

Antibiotics Class	Compound	Antibiotic concentration (μg/kg)[1] Faeces Producer A	B	C	D	Raw milk Producer A	B	C	D	Whey Producer A	B	C	D	Fresh cheese Producer A	B	C	D	Ripened cheese Producer A	B	C	D	P-value[2] C	P
	Danofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Marbofloxacin	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Oxolinic acid	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Sulfonamide	Sulfadiazine	n.d.	**9.10 ± 5.52**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.108
	Sulfathiazole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfapyridine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamerazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfamethazine	**45.0 ± 2.20**	n.d.	**2.30 ± 0.850**	**2.10 ± 0.28**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.540
	Sulfachloropyridazine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfaquinoxaline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfametizole	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Sulfadimethoxine	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
Tetracycline	Oxytetracycline	**9.30 ± 0.60**	n.d.	**1.75 ± 2.47**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.278
	Tetracycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000
	Chlortetracycline	n.d.	**3.00 ± 4.24**	n.d.	**52.0 ± 58.4**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	< **0.001**	0.278
	Doxycycline	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	1.000	1.000

[1] The concentration of each compound is expressed as the mean ± standard deviation. Values above the LOQ are bold colored. n.d.: not detected.

[2] C: Production chain factor effect; P: Producer factor effect.

S. fradiae and acts by inhibiting protein synthesis through interaction with the 50S subunit of the bacterial ribosome. It is effective against gram-positive bacteria, some gram-negative bacteria (*Helicobacter pylori*, *Haemophilus* spp., *Pasteurella* spp., *Legionella* spp.), spirochetes, *Cryptosporidium parvum*, *Chlamydia*, and *Mycoplasma*. However, certain enteric bacteria, such as *Escherichia coli* and *Salmonella* spp., are intrinsically resistant (Alcock et al., 2023). The EMA classifies tylosin into category C, indicating that it is an antibiotic to be used with caution, considering the availability of alternatives in human medicine. It should only be considered for veterinary treatment when there are no alternatives to Category D drugs that may be clinically effective (CVMP and CHMP, 2020). Sulfonamides are broad-spectrum synthetic compounds that act by inhibiting dihydropteroate synthase, which catalyses the conversion of *p*-aminobenzoic acid to dihydropteroic acid as part of the tetrahydrofolic acid biosynthetic pathway. This acid is essential for the synthesis of folate, a precursor of nucleotides and amino acids (Alcock et al., 2023). Sulfonamides are commonly used in veterinary medicine as antibacterial compounds to treat livestock diseases, such as gastrointestinal and respiratory tract infections (Ovung & Bhattacharyya, 2021). They are classified as category D (CVMP and CHMP, 2020). Finally, the quinolone enrofloxacin is a broad-spectrum compound that acts against gram-positive and gram-negative bacteria by interacting with topoisomerase II (DNA gyrase) to disrupt bacterial DNA replication, damage DNA, and cause cell death (Alcock et al., 2023). It is used to control bacterial infections in the respiratory and gastrointestinal tracts and mastitis in cattle. The EMA classifies it into category B; consequently, it is an antimicrobial of transcendental importance in human medicine, and its use in animals must be limited to mitigate the risk to public health. This should only be considered if there are no Category C or D alternatives (CVMP and CHMP, 2020).

The prevalence of antibiotics varied significantly according to the sample type, with faeces exhibiting the highest concentrations (Table 2). Chlortetracycline dominated, ranging from 3.00 ± 4.24 to 52.0 ± 58.4 μg/kg, followed by sulfamethazine (2.10 ± 0.28 to 45.0 ± 2.20 μg/kg) and, in lower concentrations, oxytetracycline (1.75 ± 2.47 to 9.30 ± 0.60 μg/kg), sulfadiazine (9.10 ± 5.52 μg/kg), and enrofloxacin (2.05 ± 2.90 μg/kg). These concentrations were found to be below the LODs of the Charm KIS test (Supplementary Table 1), potentially explaining the observed discrepancy between the commercial and analytical methods (Table 2 and Fig. 1). Developing specific screening tests for faecal samples could enhance stakeholders' ability to determine when an animal no longer harbours a particular compound, thereby preventing its transfer to the environment and the food chain (Virto et al., 2022; Zhang et al., 2022). The identified compounds have been previously identified in livestock faeces (Berendsen et al., 2015; Virto et al., 2022), including sheep (Peng et al., 2022), confirming the excretion of certain antibiotics through faeces in food-producing animals (Virto et al., 2022). Approximately 70–90 % of administered compounds, whether in their unmetabolized form or as active metabolites, have been reported to be present in animals' faeces and urine (Phares et al., 2020). These findings are particularly significant since the faecal samples obtained originated from healthy herds that had not undergone antibiotic treatment. Consequently, the transfer of antibiotics from manure to the food chain poses potential risks to human health (Zhang et al., 2022). Tetracyclines, quinolones, sulfonamides, and macrolides have been frequently detected in manure (Kuppusamy et al., 2018; Virto et al., 2022).

Notably, there were large differences in the antibiotics detected in the faeces among the producers, although this difference was not statistically significant ($P > 0.05$) (Table 2). For example, sulfamethazine was detected in all samples except those from producer B, whereas chlortetracycline was detected in faeces from producers B and D, oxytetracycline was detected in faeces from producers A and C, and sulfadiazine and enrofloxacin were only detected in the faeces of producer B. Moreover, a clear distinction was also noted in the concentrations of the detected compounds. For instance, sulfamethazine was about 45.0 ± 2.20 μg/kg in samples from producer A, while it was considerably lower in samples from producer C (2.30 ± 0.850 μg/kg) and D (2.10 ± 0.283 μg/kg). The concentration of chlortetracycline was markedly higher in the samples of producer D (52.0 ± 58.4 μg/kg) than in those of producer B (3.00 ± 4.24 μg/kg). This would be indicative of the different herd management practices and, specifically, the use of antibiotics between farms, as reflected in the cross-sectional survey (Section 3.1). These results agree with those of previous studies. For example, Peng et al. (2022) have reported on the abundance of tetracyclines, sulfonamides, quinolones, and macrolides in faecal samples of different sheep herds from China.

It is essential to analyse the presence of antibiotic residues in raw milk to avoid negative effects on consumer health, both direct effects due to the toxicology of the compounds and indirect effects due to the development of resistant microorganisms (Virto et al., 2022). In the analysed raw milk samples, the results revealed that of all the antibiotics tested by LC-MS/MS, only tylosin was detected at a concentration that varied from 6.15 ± 12.3 to 6.98 ± 8.24 μg/kg (Table 2). However, in all samples, the concentrations were below the MRLs (Brocca & Salvatore, 2023), indicating no risk to consumer health. Nonetheless, several studies have shown that antibiotics, even at concentrations below the MRLs, can favour the development of resistant bacteria (Virto et al., 2022). Furthermore, concentrations below MRLs have also been reported as affecting the activity of starter cultures; for example, a 50 % reduction in the activity of *Streptococcus* sp. (Quintanilla et al., 2019b). Therefore, antibiotic residues can affect cheese microbiota (Quintanilla et al., 2019b), and considering their importance for cheese quality and safety (Santamarina-García et al., 2022), they can compromise the final characteristics of cheese, such as the aroma or presence of pathogenic bacteria (Quintanilla et al., 2019b; Santamarina-García et al., 2022). Information on the occurrence of tylosin in raw ewe milk is scarce, but it has been previously detected in milk derived from the Awassi and Merino breeds (Richards et al., 2022). The penetration of macrolides into tissues, milk, and blood is relatively fast, with high systemic availability. Thus, they show good penetration and distribution in the udder (Richards et al., 2022), which explains the presence of tylosin in milk (Table 2).

The raw milk samples revealed the presence of tylosin exclusively in samples obtained from producers A and C. Small variations in the concentration of this antimicrobial were noted between samples from both producers, as outlined in Table 2. These findings suggest disparities in herd management and antibiotic administration practices across farms, as outlined in the accompanying survey (Table 1). It is noteworthy that the existing literature lacks information regarding distinctions or similarities in the occurrence of antibiotic residues in dairy products among producers sharing the same PDO, thus underscoring the novelty of these results.

The LC-MS/MS results (Table 2) partially agreed with those of the Eclipse Farm^{3G} test (Fig. 1A). In the raw milk sample S4 from producer A, tylosin was detected at a concentration of 24.6 μg/kg, while in the samples S3 and S4 from producer C the concentration was lower (16.1 μg/kg and 11.8 μg/kg, respectively). Considering that the LOD of this test for tylosin is 25 μg/kg, the result of close to the LOD for producer A and not for producer C would be explained. However, the 75.0 % (3/4) of the samples from producer D also yielded results close to the LOD, in which no antibiotics were detected. Similar results have been reported in the literature as false positives (Chiesa et al., 2020), which have been attributed to different substances present in milk, such as bacteriocins or lysozymes, which inhibit the growth of *Geobacillus stearothermophilus* (Yamaki et al., 2006). Nevertheless, unlike chromatographic methods, screening tests also detect metabolites or degradation compounds of antibiotics, which are essential because they maintain antimicrobial activity (Serrano et al., 2022). Consequently, the obtained results could be related to antibiotics or metabolites of antibiotics not analysed by LC-MS/MS, which have not been reported so far for dairy products.

Antibiotic residues are concentrated during the cheese-making process, although this depends on the compound and its characteristics

(Quintanilla et al., 2019b). In this sense, throughout Idiazabal cheese-making process, tylosin was the only compound detected by LC-MS/MS in whey samples, although at a slightly lower average concentration than in milk (from 5.35 ± 10.7 to 6.28 ± 7.39 μg/kg) (Table 2). In contrast, no compounds were detected in the fresh cheese samples, including the sulfonamides, quinolones, and tetracyclines identified in the faeces (Table 2). The manufacturing process of producer A led to the elimination of 3.2 μg/kg of tylosin, while for producer C it was only 1.4 μg/kg. That is, the tylosin concentration was reduced from milk to whey by approximately 14 % in the case of producer A and by approximately 29 % in case of producer C [The large differences in the cheese making settings observed in producers A and C (Supplementary Table 4), which will be discussed a bit later, must have played a role in obtaining these quite different values]. Notably, the distribution of antibiotics during cheese-making has been reported to be dose-independent (Giraldo et al., 2022), which indicates the extrapolation potential of these results to other similar cheese-making processes. The partitioning from milk to cheese or whey depends not only on the solubility of the compound but also on its ability to interact with the protein and/or fat fraction (Quintanilla et al., 2019a) and other factors to be studied yet (Giraldo et al., 2022). These results agree with what has been reported by Giraldo et al. (2022), who observed during the elaboration of a goat cheese that 75.3 % of the tylosin present in milk was eliminated through the whey, while the remaining 24.7 % was retained in the cheese. Therefore, the concentrations retained in the fresh cheese samples analysed in this study were low and undetectable by the LC-QqQ-MS/MS method (<LOD). Overall, further studies are required to elucidate the effects of cheese-making settings on antibiotic concentrations.

Although the transfer of antibiotics from milk to whey is considered beneficial for cheese safety (Giraldo et al., 2022), it is also important to consider whey safety. Dairy whey has various applications, such as fertiliser in agriculture and human and animal food because of its nutritional value (Virto et al., 2022). Sheep milk whey has a higher total nitrogen/dry matter ratio than bovine whey, doubling the soluble protein content (Carvalho et al., 2013). Whey protein processing has also been used for therapeutic purposes to obtain bioactive peptides with antioxidant, antihypertensive, antithrombotic, antimicrobial, and antiviral activities (Carvalho et al., 2013). However, there is no information on the effect of whey treatment on antibiotic concentrations.

Throughout the cheese-making process, clear differences were also observed among producers, detecting tylosin only in whey samples from producers A and C and below the MRL limits (Table 2). However, this difference was not statistically significant ($P > 0.05$). The cheese-making process and its conditions affect the concentration of antibiotics and their transfer to curds or whey (Quintanilla et al., 2019a). In case of producers A and C, large differences were observed in the cheese-making settings (Supplementary Table 4), with significant differences in the temperatures used or in the duration of treatments, such as pressing ($P \leq 0.05$). Consequently, these parameters could have affected antibiotic concentrations (Quintanilla et al., 2019a). Specifically, heat treatments that take place during milk processing, such as pasteurisation or sterilisation, have been related to the degradation of the antibiotics present in milk (Roca et al., 2011), even though degradation depends on the treatment conditions (lower temperature and time, less degradation) and the compound (for example, macrolides such as erythromycin are quite thermosensitive, whereas quinolones are very thermostable) (Quintanilla et al., 2019a). In this regard, Quintanilla et al. (2019a) have reported a maximum of 30 % degradation of antibiotics in spiked goat milk, and Gajda et al. (2017) have informed a reduction below 19 % in tetracyclines in cow milk. Milk pasteurisation does not occur in raw milk cheeses, including Idiazabal cheese (Santamarina-García et al., 2022) and there is no information in the literature on the extent to which the different stages of the cheese-making process could affect antibiotic concentration, which could be useful information for producers.

Comparing the results of the Eclipse Farm^{3G} test and the LC-MS/MS method throughout the cheese-making process, both methods were in accordance with the whey samples. The whey sample from producer A that gave a result close to the LOD by means of the screening test, had a concentration of 21.4 μg/kg of tylosin by LC-QqQ-MS/MS, being close to the LOD of the test (25 μg/kg). However, the samples from producer C presented lower concentrations (10.8 and 14.3 μg/kg) and therefore yielded negative results. For fresh cheeses, a discrepancy was observed because no compound was detected by LC-QqQ-MS/MS in the samples that gave a positive or close to LOD result. This discrepancy could be related to antibiotics not being analysed, metabolites of antibiotics not detected by the LC-QqQ-MS/MS technique used, as aforementioned (Serrano et al., 2022), or to the above-mentioned false positives due to free fatty acids, bacteriocins, and lysozyme in a minority of cheese samples produced from negative milk samples.

During cheese ripening, no antibiotics were detected by LC-MS/MS (Table 2). Cheese ripening has been reported to degrade antibiotics that may appear in fresh cheese, although this depends on the compound and its characteristics (Cabizza et al., 2017; Quintanilla et al., 2019a; Quintanilla et al., 2019b). For example, Quintanilla et al. (2019b) have observed a 95 % of oxytetracycline reduction during ripening and of only 30–45 % for quinolones, attributed to their stability at refrigeration temperatures. However, contradictory results have been reported in the literature. For example, Cabizza et al. (2017) have only observed a 17 % reduction in oxytetracycline concentration. This has been attributed to different types of milk (sheep and goat) and ripening conditions (such as acidification, ripening time, and ripening conditions) (Quintanilla et al., 2019b). As for fresh cheese samples, LC-MS/MS results did not agree with the screening test (Fig. 1), which could not only be related to false positives but also highlights the need to improve the confirmatory methods used, as stated before for meat samples (Serrano et al., 2022).

Using multivariate analysis, PERMANOVA confirmed the effect of the production chain or sample type on the occurrence of antibiotics ($P \leq 0.01$). Through PCA (Fig. 2A-B) and OPLS-DA models (Fig. 2C-D), despite its limitations, the distinction among sample types was confirmed, with faeces related to most antibiotic compounds, raw milk and whey related to tylosin, and fresh and ripened cheese samples without relation to any compound. Differences among samples, according to the manufacturer, were also observed.

3.4. Health risk assessment

To determine the health risks posed by the presence of antibiotic residues to human health, the FSM value was calculated in samples where antibiotic residues were found (Fig. 3). The FSM makes it possible to assess whether the margin between the exposure at an estimated daily intake (EDI) and the safety threshold as an acceptable daily intake (ADI) for the food hazard in question is sufficient (Quintanilla et al., 2019a). According to the results obtained, an average FSM value of 1.00 ± 0.00143 was obtained for tylosin in raw milk and whey, with the lowest value found for children (0.999 ± 0.00143). For the remaining compounds, whether concentrations similar to those found in faeces would have been maintained in raw milk and whey, the lowest FSM values would correspond to 0.945 for chlortetracycline and 0.983 for enrofloxacin in children. Nevertheless, differences in the FSM values for all antibiotics among the age categories (adults, teenagers, and children) were scarce and, consequently, were not significant ($P > 0.05$). According to the producer, differences were observed in the FSM values in milk and whey ($P \leq 0.001$) owing to the occurrence of different antibiotics along the production chain. Multivariable analysis confirmed the effect of the producer ($P \leq 0.001$), but not that of the age category ($P > 0.05$). PCA and OPLS-DA models confirmed these results (Supplementary Fig. 1). Therefore, in general, the FSM of all samples were close to 1, indicating that the consumption of raw milk or whey would not pose a significant health risk. These results agree with those reported by Quintanilla et al. (2019a), who observed that children were the most sensitive group, followed by adolescents and adults. This was mainly due to the EDI formulation, as exposure is inversely proportional to body

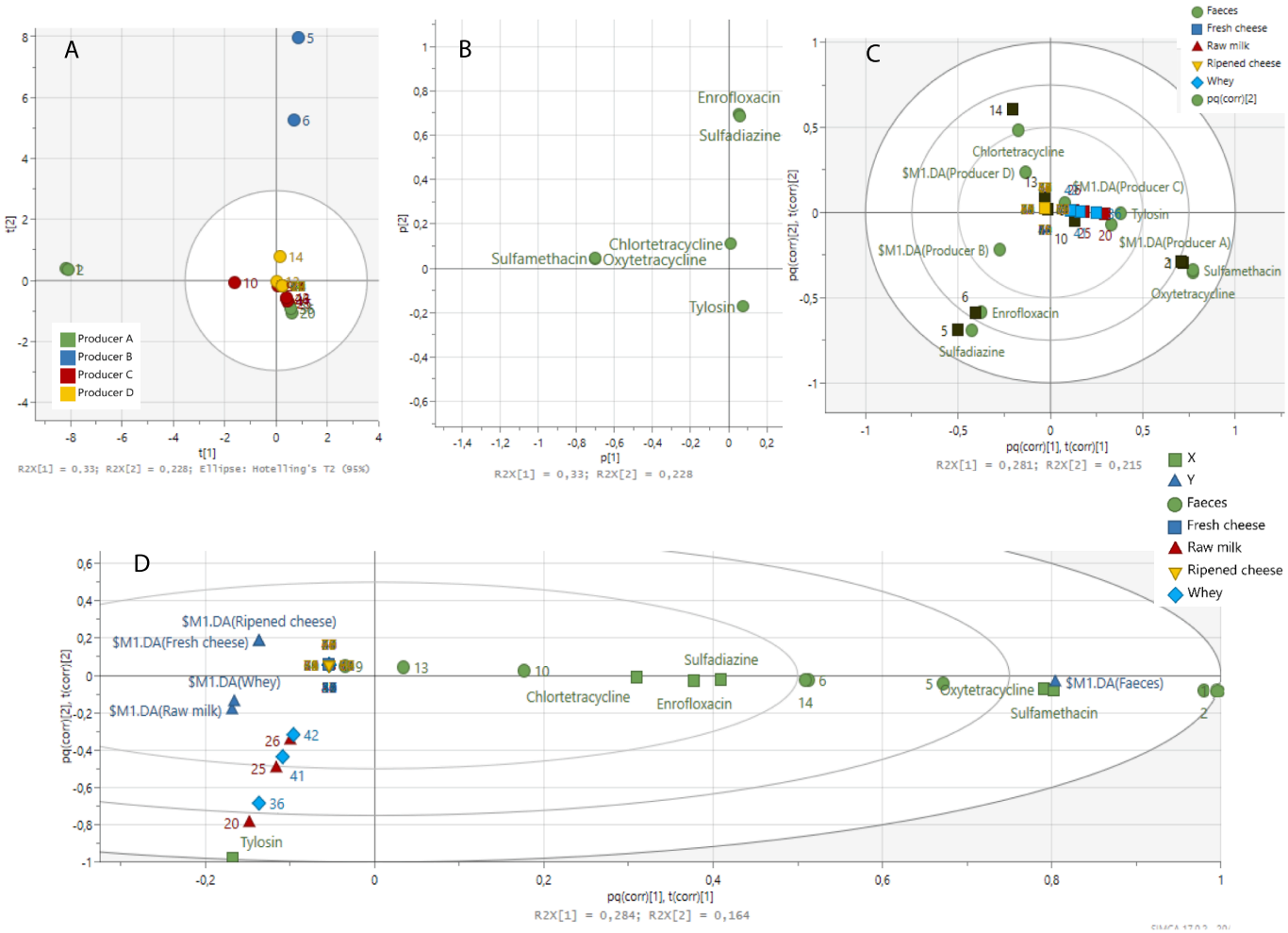

Fig. 2. PCA (A and B, scores and loadings, respectively) and OPLS-DA models (C and D, scores and loadings biplots based on the producer and production chain factors, respectively) based on the UHPLC-QQQ-MS/MS and LC-QQQ-MS/MS results.

weight (Quintanilla et al., 2019a). Quintanilla et al. (2019a) have observed for pasteurised goat's milk and cheese that macrolides, together with quinolones, have the lowest safety margins and present a greater probability of exceeding the safety margin, while β-lactams or tetracyclines present the highest safety margin. This discrepancy in the results is mainly due to the concentration of antibiotics (Virto et al., 2022).

4. Conclusion

This study elucidates antibiotic utilization in artisanal PDO dairies and the presence of residues throughout the raw milk cheese production chain. The results expose a lack of producers awareness regarding antibiotic utilization, specifically noting inconsistencies between declared and detected antibiotics. Screening tests identified samples, primarily raw milk and whey, near the LOD, with positive samples concentrated in fresh and ripened cheeses, suggesting a concentration effect during cheese-making. Chromatographically, diverse compounds were identified in faeces, including chlortetracycline and sulfamethazine. The low concentrations and high LODs in the screening test accounted for negative results. However, only tylosin was detected in milk and whey, while no antibiotics were found in fresh or ripened cheese. Positive or near-LOD results in screening tests, contrasted with non-detectable (n.d.) results by chromatography, were attributed to compounds or degradation metabolites not covered by chromatography. This underscores the potential of screening tests for stakeholders but also emphasizes their limitations, particularly when results are influenced by free fatty acids, bacteriocins, or lysozyme, for example. The study underscores the imperative for producer training, enhanced screening and analytical techniques, and the establishment of legal limits for all dairy products to prevent residue dissemination and safeguard consumer health. Furthermore, addressing the challenge of measurement techniques improvement is crucial, necessitating the development of screening tests with lower false positive rates. This entails devising more accurate tests for detecting antibiotic residues, unaffected by other compounds in milk or cheese, or implementing broad-spectrum analytical techniques for the swift identification of all antibiotic compounds and metabolites.

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CRediT authorship contribution statement

Gorka Santamarina-García: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Resources, Software, Validation, Visualization, Writing – original draft. **Gustavo Amores:** Conceptualization, Data Curation, Formal Analysis, Investigation,

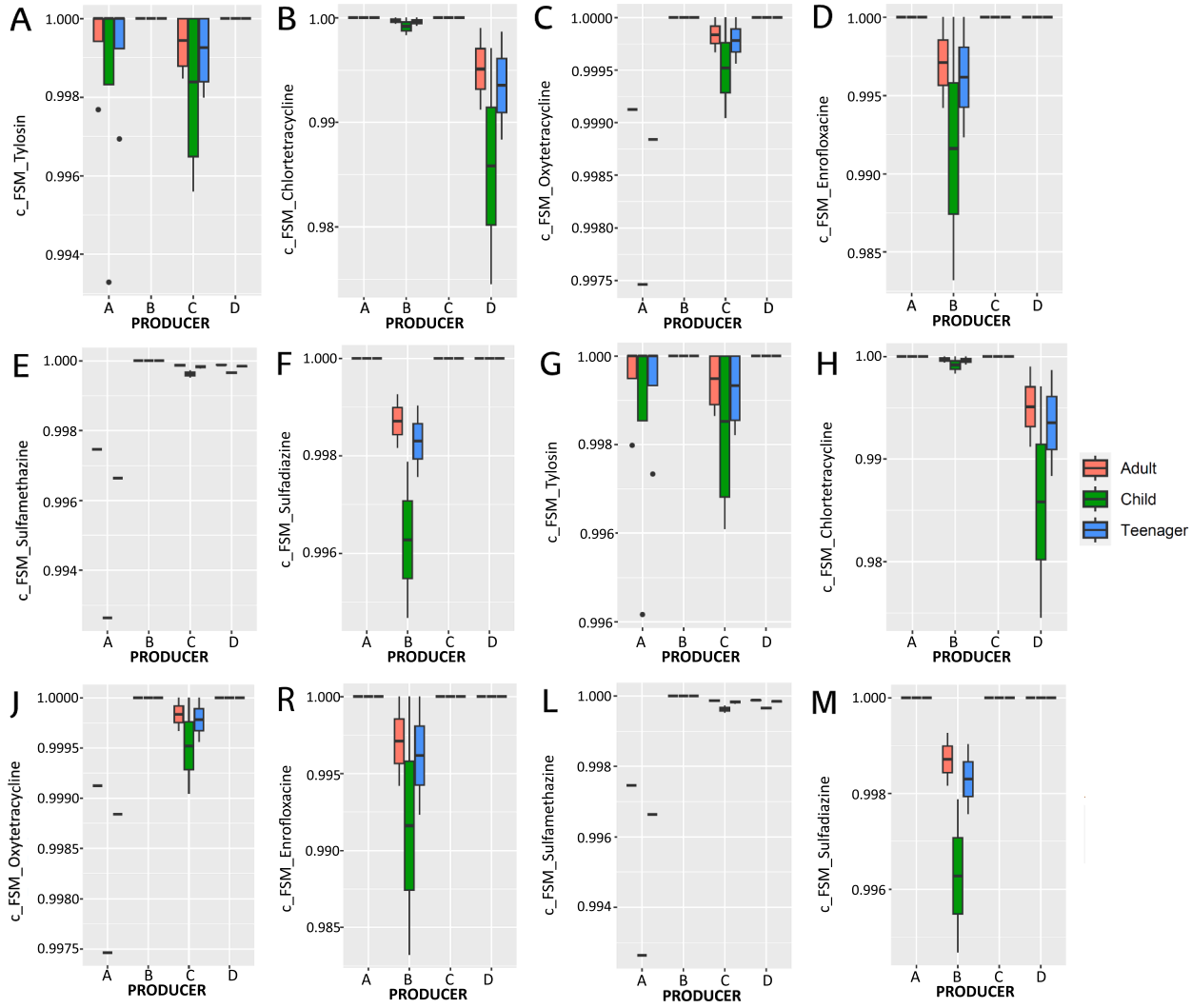

Fig. 3. Box plot representation of the FSM value calculated for adults, children and teenagers according to the analysed raw milk (A-F) and whey (G-M) samples.

Methodology, Resources, Supervision, Validation, Writing – review & editing. **Nagore Gandarias:** Data curation, Investigation. **Igor Hernández:** Conceptualization, Methodology, Resources, Writing – review & editing. **Mailo Virto:** Conceptualization, Funding acquisition, Methodology, Project administration, Resources, Supervision, Validation, Writing – review & editing.

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.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.foodchem.2024.138445.

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