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Antibiotic resistance in piedmontese and ligurian dairy production

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ABSTRACT

Background

The dissemination of antibiotic resistance genes (ARGs) through the food chain represents a major threat to public health. Dairy products from raw milk containing lactic acid bacteria (LAB) resistant to antimicrobials may serve as vectors for the transfer of resistance to commensal or potentially pathogenic bacteria in the human gut.

Objectives

This study aimed to investigate the presence of ARGs in dairy products and milk and assess the antibiotic susceptibility of LAB strains isolated from diary products made from raw milk.

Methods

41 dairy products (21 cheeses, 16 milks and 4 curds) were analysed. Antimicrobial residues were assessed using Delvotest, lateral flow rapid tests, and LC-HRMS. The presence of resistance genes related to tetracyclines, β-lactams, quinolones and erythromycin was examined using six multiplex PCR assays. The isolated LAB strains were identified by MALDI-TOF and subsequently evaluated for antibiotic resistance through the detection of the resistance genes described above, as well as by phenotypic methods, including the Kirby-Bauer disk diffusion test and determination of the minimum inhibitory concentration (MIC) for the main classes of antibiotics employed in both medical and veterinary practice.

Results

All dairy samples tested for antibiotic residues were within legal limits. ARGs were detected in all food matrices, most frequently against tetracyclines (96%), erythromycin (66%), β-lactams (55%), and quinolones (38%). Among isolated LAB strains, 20 out of 58 (34.5%) harbored at least one ARG, predominantly tetracycline resistance genes (80%), followed by β-lactams (25%), erythromycin (20%), and quinolones (5%).

Conclusions

This study confirmed that LAB present in dairy products can function as reservoirs of ARGs, representing a potential source for horizontal gene transfer within microbial ecosystems in foods and, ultimately, in the human gut. The results emphasize the importance of continuous monitoring of antimicrobial resistance in LAB and the implementation of preventive measures to limit the dissemination of resistance through dairy production and consumption.

CHAPTER I – BACKGROUND

The microbiota is defined as the population of microorganisms that colonizes a specific ecological niche, which may include the environment, animals, agri-food products, or the human host. The structure and dynamics of the microbiota within the food system can significantly affect food quality and safety; therefore, an in-depth understanding of the microbiota, potentially encompassing foodborne pathogens, is of fundamental importance for addressing and preventing potential food safety issues (Cocolin et al., 2015; Zotta et al., 2021). A wide variety of foods are produced through microbial fermentation processes, each characterized by a specific microbiota that depends on the nature of the raw materials, the production environment, and the conditions of processing and storage. Dairy products, in particular, can be regarded as complex ecosystems influenced by multiple factors, including physicochemical properties, environmental conditions, and the composition of the microbial flora (Parente et al., 2020). In this context, Next Generation Sequencing (NGS) technologies hold great potential in the field of food safety. When integrated with conventional microbiological approaches, NGS can serve as a powerful tool to investigate and thoroughly characterize the microbiota of traditional dairy products. Among the microorganisms involved in the production of fermented foods, lactic acid bacteria (LAB), either naturally present in raw materials or deliberately added as starter and/or protective cultures, are widely used in the manufacture of various food products. LAB are generally recognized as safe for human health and are therefore employed in food transformation and preservation. They inhibit undesirable and pathogenic microflora and exhibit acidifying, flavoring, and probiotic properties; consequently, there is growing interest in selecting strains with specific technological characteristics. In particular, LAB are capable of producing antibacterial compounds such as organic acids, hydrogen peroxide, antimicrobial enzymes, and bacteriocins. Bacteriocins are ribosomally synthesized proteinaceous compounds produced by both Gram-positive and Gram-negative bacteria, exhibiting inhibitory activity against bacterial strains that are closely related but distinct from the producer strain (Mohankumar et al., 2016). Research on LAB-produced bacteriocins has expanded over recent decades, aiming to employ them as natural preservatives in the food industry and to meet the increasing consumer demand for products free of synthetic preservatives and additives. Although bacteriocins have been identified in a wide range of microorganisms, special attention has recently been devoted to those produced by LAB, belonging to the genera *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc*, which can inhibit the growth of foodborne pathogens such as *Staphylococcus aureus*, *Listeria monocytogenes*, and *Bacillus cereus*. Therefore, the isolation of autochthonous LAB strains from the

Piedmont and Liguria regions capable of producing bacteriocins and effectively inhibiting spoilage and pathogenic bacteria could represent a valuable strategy for local producers and processors. Such an approach would allow the preservation of product typicity while employing natural preservative agents. Moreover, the increasing incidence of bacterial resistance to most traditional antibiotics has underscored the need to assess the occurrence and impact of antibiotic resistance in bacteriocin-producing LAB. Recent studies have demonstrated that LAB can potentially transfer antibiotic resistance genes through plasmids and transposons (Doyle et al., 2013). Plasmids are common in *Lactococcus*, *Pediococcus*, *Leuconostoc*, and *Streptococcus* species, and are also present in certain *Lactobacillus* species, while conjugative transposons have been described in *Lactococcus* and *Streptococcus*. To ensure consumer safety, it is therefore essential that LAB strains used in the food industry do not harbor transferable antibiotic resistance genes. Further investigation of these aspects is indispensable, as repeated human exposure to antibiotic-resistant LAB may pose a tangible health risk.

CHAPTER II – AIMS

The structure and dynamics of food-associated microbiota play a crucial role in determining both the safety and quality of food products. A comprehensive understanding of these microbial communities is therefore essential for the prevention and management of potential food safety issues (Cocolin et al., 2015; Zotta et al., 2021). Dairy products, in particular, represent complex ecosystems shaped by physicochemical properties, environmental factors, and the composition of the resident microbial flora (Parente et al., 2020). In this context, the combination of Next Generation Sequencing (NGS) technologies with classical microbiological methods offers a powerful and innovative approach for the detailed characterization of food microbiota. Lactic acid bacteria (LAB) are key players in many dairy fermentations, with genera such as *Lactobacillus*, *Enterococcus*, *Pediococcus*, and *Leuconostoc* spp. known for producing bacteriocins, which are proteins with bactericidal activity (Mohankumar et al., 2016). However, the potential for horizontal gene transfer of antibiotic resistance (AR) genes via mobile genetic elements such as plasmids and transposons raises concerns that LAB could act as reservoirs of AR genes, potentially transferable to humans (Doyle et al., 2013). While safety and efficacy are fundamental requirements for the application of LAB in the food industry, only a limited number of studies have monitored the presence and spread of AR genes in LAB isolated from fermented foods. The thorough characterization of the microbiota of traditional dairy products, together with their LAB component, is therefore a topic of significant scientific interest. Previous studies on *Lactobacillus* strains have extensively demonstrated their immunomodulatory potential (Rocha-Ramírez et al., 2017; Rocha-Ramírez et al., 2021); however, only a few investigations have addressed the immunomodulatory properties of individual bacteriocins (Małaczewska et al., 2021). Building on these considerations, the present project aims to characterize the microbiota of selected traditional dairy products using NGS approaches, highlighting differences between individual products from the perspective of food safety. Additionally, bacteriocin-producing LAB strains isolated from these products will be examined for their antibacterial activity against foodborne microorganisms. The characterization of LAB will also focus on the detection of AR genes to evaluate potential consumer exposure to antibiotic-resistant strains.

The present study was designed to test the following working hypotheses:

1. Traditional raw milk cheeses produced without industrial starter cultures host distinct and product-specific microbial communities shaped by geographical origin and production environment
2. Bacteriocin-producing LAB isolated from artisanal dairy products exhibit variable antibacterial activity against foodborne pathogens
3. Although antibiotic resistance genes are frequently detectable in dairy food matrices, LAB represent a limited reservoir of transferable resistance genes compared to the overall microbial community
4. Selected LAB strains are capable of modulating host innate immune responses in vitro in a strain-specific manner

The project is structured into the following phases:

1. Selection and sampling of traditional dairy products, with particular emphasis on artisanal productions from the Piedmont and Liguria regions
2. Microbiological assessment of the selected products to verify hygiene and safety parameters, including the isolation and identification of LAB
3. Screening for antibiotic residues in milk and cheeses, coupled with determination of antibiotic resistance through both phenotypic and genotypic methods

4. Extraction of bacteriocins, evaluation of their in vitro antibacterial activity, physicochemical characterization, and assessment of their interactions with the immune system using cellular models
5. Metagenomic analysis via 16S rRNA gene sequencing, followed by bioinformatic interpretation of the data using specialized computational tools

The overarching objectives of this project are to:

- Characterize the microbiota of dairy products that differ in type, production process, and geographical origin
- Assess the potential use of LAB and their bacteriocins as natural antimicrobials to enhance dairy product safety
- Investigate the prevalence and dissemination of antibiotic resistance genes in bacteriocin-producing LAB
- Gain new insights into the immunomodulatory properties of bacteriocins

Through this multi-faceted approach, the project seeks to provide a comprehensive understanding of the microbial and functional characteristics of traditional dairy products, while also evaluating the potential benefits and risks associated with LAB and their bioactive compounds.

CHAPTER III - MATERIALS AND METHODS

1. Sample selection and collection

A total of 41 dairy products, including 16 raw milk samples, 4 curds, and 21 cheeses, were collected from artisanal producers in the Piedmont and Liguria regions. Sample selection was guided by a clear scientific rationale aimed at maximizing microbiological variability while preserving comparability among products. The study focused on traditional raw milk dairy products manufactured without the use of industrial starter cultures, as these conditions allow the spontaneous development of complex autochthonous microbial communities. The Piedmont and Liguria regions were selected due to their long-standing tradition of artisanal cheesemaking and the presence of small-scale dairies characterized by limited technological standardization. This context represents an ideal model to investigate the role of natural microbiota in shaping food safety, microbial ecology and functional properties of LAB. Cheeses were preferentially selected among products with short to medium ripening times in order to capture early microbiota dynamics and to facilitate direct comparison with corresponding milk and curd samples. The inclusion of both cow’s and goat’s milk cheeses further allowed exploration of the influence of animal species on microbiota composition and antibiotic resistance patterns.

To identify the cheese producers, the relevant Local Health Authorities (ASL) for each province were contacted. From each selected producer, the following samples were collected for each type of cheese:

- one cheese sample (approximately 300 g) of unripened product
- the corresponding milk sample (50 mL) used for cheesemaking

Cheese samples were collected in sterile bags, whereas milk samples were collected in sterile preservative-free bottles. All samples were transported to the laboratory under refrigerated conditions. Milk samples were frozen and later analyzed for the detection of antibiotic residues. Cheese samples were used for the isolation of LAB and, in part, analyzed to verify hygiene and safety criteria

2. Microbiological investigations of the samples, isolation and identification of LAB

2.1 Microbiological investigations

Cheese samples were collected and transported under refrigerated conditions. Analyses were performed according to food safety and process hygiene criteria. The following microbiological determinations were carried out:

- ELFA detection of *Salmonella spp.*
- ELFA detection of *Listeria monocytogenes*
- Enumeration of coagulase-positive *Staphylococci*
- Enumeration of β-glucuronidase-positive *Escherichia coli*
- Enumeration of *Enterobacteriaceae*
- Enumeration of aerobic mesophilic bacteria at 30°C

Salmonella spp.

Rapid detection of *Salmonella spp.* was performed using the ELFA method in accordance with the AFNOR BIO 12/32-10/11 (2020) protocol for cheeses made from raw milk. Under aseptic conditions, 25 g of sample were placed into a sterile stomacher bag, and 225 mL of Buffered Peptone Water (BPW) supplemented with 5% selective supplement for *Salmonella spp.* containing inhibitors for most Gram-positive and some Gram-negative bacteria were added. Samples were homogenized in a Stomacher for approximately 2 minutes and incubated at 41.5 ± 1°C for 18–24 hours. After incubation, 1 mL of the enrichment broth was transferred into 10 mL of pre-warmed SX2 broth (41.5 ± 1°C) and further incubated at the same temperature for 6–8 hours. Subsequently, 2–3 mL aliquots of enrichment broth were heat-treated at 95–100°C for 5 minutes. After cooling, 0.5 mL of each sample was analyzed using the ELFA (Enzyme-Linked Fluorescent Assay) method with a VIDAS instrument (bioMérieux). Positive samples were confirmed by isolation from BPW or SX2 broth following ISO 6579:2017. Biochemical confirmation of presumptive *Salmonella spp.* strains (Rapid 20E, bioMérieux) was followed by serological typing using commercially available polyvalent and monovalent antisera.

Listeria monocytogenes

Detection of *Listeria monocytogenes* was performed using a rapid ELFA method according to AFNOR BIO 12/27-02/10 (2020). Twenty-five grams of sample were diluted 1:10 in LMX pre-enrichment broth (without supplement), homogenized, and incubated at 37°C for 20–24 hours. Subsequently, 3 mL of culture were transferred into 6 mL of LX enrichment broth and incubated for an additional 6–8 hours at 37°C. After incubation, samples were screened using the ELFA method with a VIDAS instrument (bioMérieux). Positive samples were confirmed by isolation from LX broth according to ISO 11290-1:2017, and biochemical identification was carried out using API Listeria (bioMérieux).

Coagulase-Positive *Staphylococci*

Enumeration of coagulase-positive *Staphylococci* was carried out in accordance with ISO 6888-2:2021. Ten grams of sample were mixed with 90 mL of Buffered Peptone Water (BPW). Serial tenfold dilutions were prepared up to 10^{-4}. From dilutions 10^{-3} and 10^{-4}, 1 mL of each was transferred into two sterile Petri dishes. Pour plating was performed using Baird-Parker agar supplemented with Rabbit Plasma Fibrinogen (RPF), poured to a depth of approximately 3 mm. Plates were allowed to solidify on a level, cool surface, inverted, and incubated at 37 ± 1°C for 18–24 hours. *Staphylococci* formed black or grey colonies surrounded by a clear halo indicative of coagulase activity.

β-Glucuronidase-Positive *Escherichia coli*

Enumeration of β-glucuronidase-positive *E. coli* was performed according to ISO 16649-3:2015. Under aseptic conditions, 10 g of sample were mixed with 90 mL of BPW and homogenized for approximately 2 minutes in a Stomacher. Serial dilutions (10^{-1}, 10^{-2}, and 10^{-3}) were prepared. From each dilution, 1 mL was plated by pour plating with Tryptone Bile X-glucuronide (TBX) agar (cooled to 44–47°C), to a depth of approximately 3 mm. Plates were allowed to solidify and incubated at 44 ± 1°C for 18–24 hours. Characteristic colonies appeared blue-green.

Enterobacteriaceae

Enumeration of *Enterobacteriaceae* was performed according to ISO 21528-2:2017. Twenty-five grams of sample were mixed with 225 mL of sterile Buffered Peptone Water (Oxoid) for pre-enrichment. After homogenization, 1 mL of each serial dilution was plated in duplicate with approximately 15 mL of Violet Red Bile Glucose (VRBG) agar, tempered at 44–47°C. The inoculum and medium were mixed gently and allowed to solidify. Plates were incubated aerobically at 37°C for 24 hours. After incubation, plates with fewer than 150 colonies were counted. Typical colonies—pink to red or purple, sometimes surrounded by a precipitated halo—were confirmed by oxidase testing and glucose fermentation (O/F medium incubated at 37°C for 24 h). Colonies that were oxidase-negative and glucose-positive were confirmed as *Enterobacteriaceae*.

Aerobic Mesophilic Count at 30°C

Enumeration of total aerobic mesophilic bacteria was performed according to UNI EN ISO 4833:2013. Ten grams of sample were homogenized in a stomacher bag with 90 mL of pre-warmed BPW (30 ± 1°C). Serial tenfold dilutions were prepared. From each dilution, 1 mL was plated in duplicate using Milk Plate Count Agar (MPCA), cooled to 44–47°C. Plates were mixed gently, allowed to solidify, and incubated at 30 ± 1°C for 72 hours. After incubation, plates containing fewer than 300 colonies were counted, and results were expressed as colony-forming units (CFU) per gram of sample.

2.2 Isolation, enumeration and identification of LAB

The enumeration of lactic acid bacteria (LAB) was performed on selected samples of milk, curd, and cheese (Table 1, Annex 1). Ten grams of solid samples and 10 mL of liquid samples were initially collected and mixed with 90 mL of Buffered Peptone Water (BPW). From this first 10^{-1} dilution, serial tenfold dilutions were prepared by transferring 1 mL of the previous dilution into 9 mL of peptone water. Subsequently, 1 mL of each dilution was plated into sterile empty Petri dishes, using the double-layer pour plate method. Two selective media were used for the growth of lactic acid bacteria: MRS agar and M17 agar, which promote the growth of *Lactobacillus* spp. and *Lactococcus* spp., respectively. Each dilution was inoculated onto duplicate plates of both media: one plate was incubated at 37 °C for 48 h, and the other at 30 °C for 72 h. After incubation, bacterial growth was enumerated. From the MRS and M17 plates, between one and eight colonies (depending on the observed concentration) were selected, picked, and subcultured on blood agar plates. These plates were then incubated for 24 h under microaerophilic conditions. The resulting colonies were used for strain identification. Isolates were analyzed using the MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization - Time of Flight) mass spectrometry system (BRUKER, Massachusetts, USA). This instrument operates by measuring the mass-to-charge (m/z) ratio of ions emitted from the sample, producing a characteristic spectrum that is compared with reference “fingerprint” profiles to determine species identity. The identified strains were subsequently preserved in a cryobank at -20 °C.

3. Detection of antibiotic residues in milk and cheese; determination of antibiotic resistance by phenotypic and genotypic methods

3.1 Investigation of antibiotic residues in milk and cheese

Screening analyses for the detection of inhibitory substances (antimicrobial residues) were carried out on the 16 milk samples collected (Table 1), using a panel of tests that included the Delvotest (a microbiological method with indicator, in accordance with Ministerial Decree of 26/03/1992) and two types of rapid multiresidue lateral flow assays for the identification of the main classes of molecules commonly used in the treatment of dairy cattle (β-lactams, tetracyclines, quinolones, sulfonamides, and macrolides). In addition, the 16 milk samples and 18 cheese samples at the end of ripening (Table 1) were further analyzed for the quantitative determination of inhibitory substances using advanced high-resolution mass spectrometry (LC-HRMS) techniques. Samples that tested positive were compared with the maximum residue limits established by Regulation (EU) No. 37/2010. The molecules investigated and their respective limits of quantification (LOQ), as determined by LC-HRMS analysis, are summarized in Table 2 (Annex 1).

3.2 Determination of antibiotic resistance by phenotypic methods

The antibiotic resistance profiles of 58 LAB strains identified were evaluated. Qualitative phenotypic analyses were performed using the Kirby–Bauer disk diffusion method, while quantitative analyses were carried out by broth microdilution to determine the Minimum Inhibitory Concentration (MIC).

Kirby–Bauer method

The isolates were streaked on blood agar and incubated for 24 h at 37 °C. A bacterial suspension standardized to a turbidity of 0.5 McFarland was then prepared and evenly spread on Mueller–Hinton agar supplemented with blood (for *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and

Pediococcus) and on Mueller–Hinton agar (for *Enterococcus* and *Staphylococcus*). The following antibiotic discs were applied for susceptibility testing:

- Tetracycline (30 µg)
- Gentamicin (10 µg)
- Erythromycin (15 µg)
- Ciprofloxacin (5 µg)
- Ampicillin (10 µg)
- Streptomycin (10 µg)

The inhibition zone diameters were measured using a caliper. Given the non-pathogenic nature of lactic acid bacteria, no official breakpoint values are available for the strains analyzed; therefore, standard interpretive criteria used for pathogenic bacteria could not be applied. For this reason, the results were compared with MIC values obtained by broth microdilution and supplemented with molecular analysis data. This combined approach allowed a more detailed evaluation of the resistance or susceptibility traits of the analyzed strains, despite the absence of official interpretive standards.

Broth microdilution

Growth conditions and culture media were established according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST), depending on the bacterial genus. The isolates were streaked on blood agar and incubated for 24 h at 37 °C. A bacterial suspension standardized to 0.5 McFarland was prepared; 10 µL of this suspension was inoculated into 11 mL of Cation-Adjusted Mueller–Hinton Broth with Lysed Horse Blood (for *Lactococcus*, *Lactobacillus*, *Leuconostoc*, *Streptococcus* and *Pediococcus*) or Cation-Adjusted Mueller–Hinton Broth (for *Enterococcus* and *Staphylococcus*). The broths were dispensed into Sensititre™ GPALL1F (Figure 1, Annex 2) and EULACBI1 (Figure 2, Annex 2) plates following the manufacturer's instructions. The following antibiotics were tested at the indicated concentration ranges:

Antibiotic	Abbreviation	Concentration range (µg/mL)
Ampicillin	AMP	0.12–8
Cefoxitin	FOXS	6

Antibiotic	Abbreviation	Concentration range (µg/mL)
Ciprofloxacin	CIP	1–2
Clindamycin	CLI	0.03–16
Chloramphenicol	CHL	0.12–64
Daptomycin	DAP	0.5–4
Erythromycin	ERY	0.015–8
Gentamicin	GEN	0.5–256
Kanamycin	KAN	2–1024
Levofloxacin	LEVO	0.25–4
Linezolid	LZD	1–8
Moxifloxacin	MXF	0.25–4
Neomycin	NEO	0.12–64
Nitrofurantoin	NIT	32–64
Oxacillin + 2% NaCl	OXA+	0.25–4
Penicillin	PEN	0.06–8
Quinupristin/Dalfopristin	SYN	0.5–4
Rifampicin	RIF	0.5–4
Streptomycin	STR	0.5–1000
Tetracycline	TET	0.12–64
Tigecycline	TGC	0.03–0.5
Trimethoprim/Sulfamethoxazole	STX	0.5/9.5–4/76
Vancomycin	VAN	0.25–32

The interpretation of results was based on comparison of the MIC values obtained with the official breakpoint values defined by CLSI for *Lactobacillus spp.*, *Lactococcus spp.* and *Streptococcus spp.*. For *Streptococcus spp.*, EUCAST breakpoint values were used for the interpretation of MICs of gentamicin, rifampicin and moxifloxacin. For the *Staphylococcus hominis* strain, MIC values were interpreted according to EUCAST breakpoints, except for tetracycline and clindamycin, for which CLSI values were applied.

3.3 Determination of Antibiotic Resistance by Genotypic Methods

Sample Selection

The determination of antibiotic resistance was carried out using endpoint PCR, which allows for the detection of genes encoding resistance factors. Analyses were performed both on food matrices (milk, curd, and cheese) and on strains isolated from them. A total of 87 samples were tested, including 29 food matrices (4 milk, 4 curd, and 21 cheeses) (Table 20) and 58 bacterial strains (Table 21).

DNA Extraction

For DNA extraction from food matrices, the ExtractMe Kit for genomic DNA isolation from a variety of sample sources (BLIRT S.A., Gdańsk, Poland) was used starting from 30 mg of sample, following the manufacturer's instructions. For DNA extraction from isolated strains, a single bacterial colony was resuspended in 500 µL of sterile ultrapure water and subjected to heat treatment at 99°C for 10 minutes.

DNA Amplification

Six sets of endpoint multiplex PCR assays, developed in the project "Resistome: Investigation of Genetic Markers of Antibiotic Resistance in the Dairy Supply Chain," were selected for the amplification of genes responsible for antibiotic resistance. Primers were designed to detect genes conferring resistance to major families of antibiotics currently or previously used in veterinary prophylactic or therapeutic treatments, including tet (tetracyclines), bla (β-lactams), qnr (quinolones), and erm (erythromycin), which are widely documented in the literature.

Specifically, the following were used:

- Three multiplex PCRs for the amplification of seven tetracycline resistance genes (Ng et al., 2001): I) tet(B); II) tet(A); III) tet(K), tet(L), tet(M), tet(O), tet(S);
- One multiplex PCR for the detection of two bla genes responsible for β-lactam resistance (Fang et al., 2008): IV) blaCTX-M and blaTEM;

- One multiplex PCR for the detection of three qnr genes responsible for quinolone resistance: V) qnrA, qnrB, qnrS (Salah et al., 2019);
- One multiplex PCR for the detection of three erm genes responsible for erythromycin resistance: VI) ermA, ermB, ermC (Ghanbari et al., 2016).

Target genes and primer sequences are reported in Table 3 (Supplement 1).

Multiplex PCR reactions were prepared in a final volume of 25 µL containing 12.5 µL of DreamTaq Hot Start PCR Master Mix (2x) (Thermo Scientific, Waltham, Massachusetts, USA), 1 µL of each primer pair, and 5 µL of DNA.

Amplification conditions were as follows:

- Initial denaturation: 95°C for 3 minutes
- Denaturation: 95°C for 30 seconds
- Annealing: primer-specific temperatures reported in Table 4 (Supplement 1)
- Extension: 72°C for 1 minute
- Final extension: 72°C for 10 minutes

Steps 2–4 were repeated for 35 cycles.

The amplified products were visualized by capillary electrophoresis using the QIAxcel Advanced System (QIAGEN, Hilden, Germany). The lengths of amplification products, reported in Table 4 (Supplement 1), were compared with a molecular weight marker (Bio-Rad, Hercules, California, USA).

Whole Genome Sequencing (WGS)

For an in-depth characterization of antibiotic resistance, whole genome sequencing (WGS) was performed on a selection of isolated strains. DNA was extracted using the ExtractMe Genomic DNA Isolation Kit (Blirt, Gdańsk, Poland) following the manufacturer's instructions with some modifications. Specifically, the initial lysis step included treatment with lysozyme (10 mg/mL) for 90 minutes at 37°C. DNA quantification was performed using a Qubit fluorometer (Thermo Fisher Scientific). Subsequently, libraries were prepared using the Illumina DNA Library Prep Kit (Illumina, San Diego, CA, USA) and sequenced on the Illumina MiSeq system with the MiSeq V3 Kit, generating paired-end reads of 2 × 151 bp, according to the manufacturer's protocol. Bioinformatic analysis was conducted using the Galaxy platform (Afgan et al., 2022; Tangaro et al., 2021). Raw sequences were processed with Trimmomatic 0.38 (Bolger et al., 2014) to remove Nextera adapters and other Illumina-specific sequences (Illuminaclip set to Nextera for paired-end reads), trimming low-quality bases at the start and end of reads (leading:10, trailing:10), and cutting reads when average quality scores dropped below 20 in a 4-base sliding window (sliding window: 4:20). Reads shorter than 40 bases after processing were discarded (minlen: 40). Processed reads were de novo assembled using Unicycler 0.4.8.0 (Wick et al., 2017) in bridging mode with moderate contig sizes and assembly error rates (bridging mode: Normal). Contigs shorter than 200 bp were excluded (minimum contig length: 200 bp). The assembled genomes were analyzed at the Center for Genomic Epidemiology (CGE) (https://www.genomicepidemiology.org/services/). Specifically, the ResFinder platform was used to search for resistance genes (http://genepi.food.dtu.dk/resfinder), and KmerFinder 3.2 was used to confirm bacterial species identification (https://cge.food.dtu.dk/services/KmerFinder/).

4. Extraction, Antibacterial Activity, and Characterization of Bacteriocins

For the evaluation of antibacterial activity, 40 strains of lactic acid bacteria isolated and identified were selected to examine the in vitro antibacterial activity of bacteriocins. The study included 12 strains of Lactobacillus spp., 18 of Lactococcus spp., 5 of Leuconostoc spp., and 5 of Streptococcus spp.

4.1 Bacteriocin Extraction

To prepare the CFS (Cell Free Supernatant), the selected lactic acid bacterial strains were plated on blood agar and incubated for 48 hours at 30°C. Subsequently, a standardized bacterial suspension at 0.5 McFarland was prepared, and 1 mL of this suspension was inoculated into 9 mL of MRS broth. The resulting mixture was incubated for a further 48 hours at 30°C. At the end of incubation, the broths were centrifuged to separate the cellular components. To ensure sterility, the supernatants were filtered through 0.22 µm pore filters. Finally, the CFS solutions were diluted in water to the following concentrations: 50%, 25%, 12.5%, 6.25%, and 3.125%.

4.2 In Vitro Evaluation of Bacteriocin Antibacterial Activity

For the microdilution tests, certified strains of E. coli (ATCC 25922) and S. aureus (ATCC 33862) were plated on blood agar and incubated for 24 hours at 37°C. The bacterial suspension was prepared by inoculating 10 µL of a 0.5 McFarland standardized solution into 5 mL of Mueller Hinton Broth. For the test setup, 100 µL of the bacterial suspension were inoculated into 100 µL of the CFS solutions at different concentrations (100% (undiluted), 50%, 25%, 12.5%, 6.25%, and 3.125%). Samples were incubated at 35°C for 24 hours to determine the minimum inhibitory concentration (MIC). To verify the minimum bactericidal concentration (MBC), the solutions corresponding to the lowest concentration without visible growth and the next lower concentration were plated on blood agar to assess bacterial viability.

4.3 Bacteriocin Characterization

Based on the results obtained, three strains belonging to the genus *Lactobacillus* were selected for bacteriocin characterization analyses:

- Strain 26: *Lactobacillus paracaei*
- Strain 30: *Lactobacillus plantarum*
- Strain 58: *Lactobacillus curvatus*

For heat sensitivity tests, they were thermally treated for 10 minutes at the following temperatures: 40°C, 70°C, and 100°C. To determine the effect of pH, CFS were adjusted with NaOH or HCl to reach the following pH values: 3, 6, and 9. Microdilution tests were performed as described for the pathogens E. coli (ATCC 25922) and S. aureus (ATCC 33862) at concentrations of 100% and 50%.

Immunomodulatory Effect

A selection of four lactic acid bacterial strains isolated during the project (strain 30 *Lactobacillus plantarum*, strain 26 *Lactobacillus paracasei,* strain 58 *Lactobacillus curvatus*, and strain 51 *Lacticaseibacillus paracasei*) was examined to evaluate their direct effect on a porcine intestinal cell line (IPEC-J2) after 2 hours of infection.

Infection with Bacterial Strains and Innate Immunity Modulation

To assess the direct effect of these four strains, IPEC-J2 cells, porcine intestinal cells (IZSLER Cell Bank code BS CL 205), were used; these cells spontaneously secrete IL-8 and are used to evaluate intestinal inflammatory responses (Razzuoli et al., 2013). Cells were maintained in culture in a medium composed of Dulbecco’s Minimal Essential Medium (DMEM, Euroclone) and Ham F12 (Euroclone) (1:1), 10% fetal bovine serum (FBS, Euroclone), 1% Penicillin/Streptomycin (P/S, Euroclone), and 2 mM L-glutamine (Euroclone). For the experiments, cells were seeded in 12-well plates (1 mL/well, 150,000 cells/well) and incubated at 37°C with 5% CO2 until confluence (16–22 h). The evening before the experiment, a colony of each strain was plated on De Man–Rogosa–Sharpe agar (MRS) overnight at 37°C. The next morning, a 1:10 subculture was performed (1 mL of bacterial suspension in 9 mL of Brain Heart Infusion (BHI; Sigma) for 2–3 hours at 37°C) to obtain bacteria in the logarithmic growth phase. At 3 McFarland, strains were pelleted (3’ at maximum speed) and resuspended at a concentration of 10^8 CFU/mL in DMEM/F12 medium (Euroclone) without FBS or P/S. Cells were incubated at 37°C in 5% CO2 for 2 hours with 1 mL of bacterial suspension. For each bacterial strain, five wells of IPEC-J2 cells were infected; cells treated with medium only without FBS and P/S were used as control. At the end of infection, cells were washed four times with medium without FBS and P/S and re-incubated in complete culture medium at 37°C in 5% CO2 for 3 hours. Supernatants were stored at −80°C for potential ELISA analyses, and RNA was extracted from the cells. Three independent experiments were conducted.

Innate Immunity Modulation

The modulation of selected gene expression was evaluated after two hours of infection of IPEC-J2 cells with each selected strain. To assess the modulation of Interleukin 6 (IL6), C-X-C Motif Chemokine Ligand 8 (CXCL8), IL18, Tumor Necrosis Factor alpha (TNFα), Transforming Growth Factor beta (TGFβ1), NFKb/p65, Toll-like receptor (TLR)4, TLR5, cyclic GMP-AMP synthase (cGAS), interferon regulatory factor (IRF)3, and interferon beta (INFβ), total RNA was extracted from IPEC-J2 cells using the RNeasy Mini Kit (Qiagen) following the manufacturer’s instructions. 1000 ng of RNA were reverse transcribed using the iScript cDNA Synthesis Kit (Biorad) according to the manufacturer’s instructions, and RT-qPCR was performed as previously described using a CFX96™ system (Biorad) (Razzuoli et al., 2022) with primers listed in Table 5. Porcine glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as the housekeeping gene. Expression of selected genes was calculated using the 2^-ΔΔCq formula, where ΔΔCq = ΔCq (treated) - ΔCq (control), and ΔCq values were obtained using ΔCq = Cq (target gene) - Cq (reference gene). A Cq value of 39 was used as the threshold in PCR tests (samples were considered positive if Cq <39).

Bacterial Invasion

The evening before the experiment, a colony of *Lactobacillus curvatus* strain 58 and a colony of *Salmonella typhimurium* (slm) ATCC14028 were grown in BHI overnight at 37°C. Confluent IPEC-J2 cells were infected with 1 mL of bacterial suspension of a single tested strain (strain 58 *Lactobacillus curvatus*) at a concentration of 10^8 CFU/mL and incubated at 37°C in 5% CO2 for 2 hours. After removing the bacteria, cells were washed four times with medium without FBS and P/S and then infected with 1 mL of slm bacterial suspension at 10^8 CFU/mL, incubated at 37°C in 5% CO2 for 1 hour. Subsequently, cells were washed four times with medium without FBS and P/S and treated with 300 µg/mL colistin (Microbiol & C. s.n.c.) in medium without FBS and P/S at 37°C in 5% CO2 for 1 hour to remove all extracellular bacteria. Cells were then washed three times with medium without FBS and P/S and lysed by adding 200 µL of 1% Triton X-100 (VWR) in phosphate-buffered saline (PBS, VWR) at room temperature for 10 minutes. 800 µL of PBS were added to each well; the resulting cell suspension was serially diluted in PBS and plated on XLD agar plates (Sigma) (Schmidt et al., 2008) and incubated at 37°C for 48 hours. Colony-forming units of slm were then counted. Cells treated with medium only were used as a negative control. The experiment was performed twice in quadruplicate.

5. Analysis by 16S rRNA Metagenomic Approach

Samples were collected from two local dairies, from which two different types of cheese were sampled. These samplings were repeated at two different times of the year (early summer and autumn) to monitor potential climate-related variations. The first dairy (Caseificio 2) provided raw milk, curd, and Maccagno cheese at two different ripening stages. The second dairy (Caseificio 3) provided raw milk, curd, and a fresh "tometta" type cheese at two different ripening stages. The samples underwent DNA extraction and purification using an Invitrogen kit (PureLink™ Genomic DNA Mini Kit REF K1820-02). The extracted DNA was stored at –20 °C pending amplification. Once the DNA was isolated and purified, amplicons of the region of interest (16S rRNA) were obtained via PCR. The PCR mixture for each sample was prepared as detailed in Table 6 (Appendix 1). Following amplification, the desired amplicons were purified using magnetic beads (NucleoMag® NGS Clean-up Size Select). After purification, the supernatant, containing the DNA of interest, was transferred to a new 96-well PCR plate, which could then be stored at –20 °C. A second PCR reaction was crucial for the addition of adapters (indices) required for subsequent sequencing. The Nextera XT Index Kit was used, which attaches two indices (i7 and i5) to each sample. The indices used, along with their respective sequences, are presented in Table 7 (Appendix 1). For validation of the gene library, a dual control approach was employed. The first control involved quantifying each sample individually using Qubit, which relies on fluorometric quantification of the library. To achieve accurate and precise quantification of dsDNA, the Qubit dsDNA HS (High Sensitivity) and Qubit dsDNA BR (Broad Range) assay kits were utilized. Once the concentration in ng/µl was obtained, it was converted to nM using the following formula:

(concentration in ng/µl) / (660 g/mol * average library size) * 10^6 = concentration in nM

Subsequently, a second validation step was performed on the pooled libraries via automated electrophoresis using the Agilent Bioanalyzer 2100 system. The Bioanalyzer High Sensitivity DNA kit was selected, allowing for reliable sizing and quantification of limited samples within the pg/µL concentration range. For DNA denaturation, 5µl of 0.2N NaOH and 5µl of the 4nM pooled sample libraries were used. For sequencing, the pool comprising the sample mix and control mix was loaded into an "Illumina" cartridge, and the sequencing process was initiated until completion.

6. Statistical analysis

Statistical analysis was performed to support the interpretation of microbiological, molecular and functional data. Given the exploratory nature of the study and the limited sample size, statistical analyses were mainly descriptive and comparative. For the purpose of comparative analyses, samples were assigned to predefined sampling groups created a priori according to matrix (milk, curd, cheese), animal species, cheese type, ripening stage and production site. All statistical comparisons were performed between these predefined sampling groups, in order to ensure consistency between experimental design and data analysis. Microbiological counts were expressed as log10 CFU/g or log10 CFU/mL. Data distribution was evaluated and, due to the non-normal distribution of most variables, non-parametric statistical tests were applied. Comparisons between two independent sampling groups (e.g. milk vs cheese, cow vs goat milk products) were performed using the Mann–Whitney U test, while comparisons among more than two sampling groups (e.g. different cheese types or ripening stages) were carried out using the Kruskal–Wallis test, followed, when appropriate, by post hoc multiple comparison tests. Categorical data, such as the presence or absence of antibiotic resistance genes and the distribution of resistant isolates among sampling groups, were analyzed using the chi-square test or Fisher's exact test when expected frequencies were low. For RT-qPCR experiments, relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. Statistical significance of gene expression differences among experimental conditions and sampling groups was assessed using one-way analysis of variance (ANOVA), followed by post hoc tests to correct for multiple comparisons. A p-value < 0.05 was considered statistically significant. All statistical analyses were performed using GraphPad Prism software.

CHAPTER IV – RESULTS

Sampling and Sample Collection

A total of nine family-run dairy farms agreed to participate in the study: seven located in the Piedmont region (four in the province of Vercelli, two in the province of Biella, and one in the province of Turin) and two in the Liguria region (one in the province of Genoa and one in the province of Imperia). These farms provided samples of cheese, curd, and milk, for a total of 41 samples (Table 1, Appendix

1). All selected cheeses met the following criteria: they were produced from raw milk, underwent short ripening periods, and were manufactured without the use of commercial starter cultures. In addition, some of the selected cheeses held the PAT (Traditional Agri-food Products) designation listed in the official register of the Piedmont Region, specifically:

- *Caprino Valsesiano*
- *Toma della Valsesia*
- *Toma di Capra*
- *Toma Biellese*

Caprino Valsesiano is a typical PAT-certified cheese from Piedmont, traditionally produced in the Valsesia area (province of Vercelli). Goat farming has been deeply rooted in this region for centuries due to its geomorphological characteristics, which in some areas limit cattle grazing. *Caprino Valsesiano* is a raw goat's milk cheese with a soft to semi-hard texture. It is consumed fresh or after short ripening, generally not exceeding 60 days. The cheese is shaped as a small cylinder weighing between 100 and 500 g.

Toma della Valsesia is a PAT-certified cheese from the Piedmont Region, produced from raw cow's milk. Its production area includes all municipalities within the Valsesia valley (province of Vercelli). The cheese undergoes short to medium ripening and has a soft, elastic texture. The wheels weigh between 1 and 3 kg.

Toma di Capra is a Piedmontese PAT cheese made from raw goat's milk. The ripening period ranges from a minimum of 15 days to a maximum of 6 months, and the cheese typically weighs between 2 and 4 kg. *Toma Biellese* is a Piedmontese PAT cheese produced in the province of Biella. It is made from raw cow's milk and undergoes medium to long ripening. It has a cylindrical shape and weighs between 3 and 8 kg.

Each participating farm produces its own milk, which is used for cheesemaking directly on site. The cheesemaking process takes place in the dairy facility attached to the farm, and subsequent ripening is carried out in the farm's own maturation room.

Microbiological Analyses

Microbiological analyses aimed at verifying hygiene and safety criteria were carried out on a total of 18 cheese samples. The microbiological results are presented in Table 8 (Appendix 1). The safety parameters *Salmonella spp.* and *Listeria monocytogenes* were not detected in any of the samples. The parameter *E. coli* β-glucuronidase tested satisfactory in 66.7% of the samples (12/18), acceptable in 27.8% (5/18), and unsatisfactory in one sample (5.6%), according to the "Guidelines for Risk Analysis in the Field of Food Microbiology" issued by the Piedmont Region (Rev. 00/2013, Annex B) and Regulation (EC) No. 2073/2005 and subsequent amendments. According to the same guidelines, coagulase-positive staphylococci were found to be satisfactory in 66.7% of the samples (12/18), acceptable in 22.2% (4/18), and unsatisfactory in two samples (11.1%). Enterobacteria counts ranged from a minimum of 9.3×10^2 to a maximum of 2.7×10^7 CFU/g, while the mesophilic aerobic count at 30 °C was high, with values ranging from 3.1×10^7 to 1.9×10^9 CFU/g. These results are consistent with the high levels of lactic acid bacteria detected in the samples.

Isolation and Identification of Lactic Acid Bacteria (LAB)

Enumeration of LAB

The enumeration of lactic acid bacteria present in the tested samples is reported in Table 9 (Appendix 1). In general, the data show that the concentration of lactic acid bacteria in milk and curd samples was lower than that observed in the cheese samples.

Identification of Isolated Colonies

Identification by the MALDI-TOF system allowed the classification of a total of 361 colonies. The detected species are summarized in Table 10 (Appendix 1). The most represented genera were *Lactococcus* (177 isolates, 49%), *Streptococcus* (41 isolates, 11%), *Lactobacillus* (36 isolates, 10%), *Enterococcus* (31 isolates, 9%), and *Leuconostoc* (26 isolates, 7%).

Selection of LAB

A total of 58 strains were selected among the colonies isolated and identified in the previous phase. The strains and the growth media from which they were obtained are summarized in Table 11 (Appendix 1). The most represented genera were *Lactococcus* (24 strains, 41%), *Lactobacillus* (15 strains, 26%), and *Leuconostoc* (8 strains, 14%).

The strains isolated in this phase were subsequently used for the determination of antibiotic resistance.

Detection of Antibiotic Residues in Milk and Cheese

All samples analyzed using the Delvotest, the rapid multiresidue lateral flow test, and LC-HRMS HPLC methods tested negative, with the exception of cheese sample no. 40, in which a concentration of 3.65 ng/g of lincomycin was detected. This value is significantly lower than the maximum residue limit (MRL) of 150 μg/kg established for milk by Regulation (EU) No. 37/2010. The results for milk samples are summarized in Table 12 (Appendix 1), while the results for cheese samples are summarized in Table 13 (Appendix 1).

Determination of Antibiotic Resistance by Phenotypic Methods

The inhibition zone diameters obtained using the Kirby–Bauer method are summarized in Table 14 (Appendix 1). All MIC (Minimum Inhibitory Concentration) values are reported in Table 15 (Appendix 1). Tables 16, 17, 18, and 19 (Appendix 1) describe the interpretation of the results for the genera *Lactobacillus spp.*, *Lactococcus spp.*, *Streptococcus spp.*, and *Leuconostoc spp.*, respectively, while Table 20 reports the interpretations for the other bacterial species. Based on the MIC values obtained, 93% (14/15) of the tested *Lactobacillus spp.* strains were resistant to vancomycin and susceptible to all other antibiotics examined. Among *Lactococcus spp.* strains, 21% (5/24) were resistant to trimethoprim/sulfamethoxazole, 12.5% (3/24) to erythromycin, and 8% (2/24) to tetracycline and clindamycin. *Streptococcus spp.* showed no resistance to any of the antibiotics tested. One *Staphylococcus hominis* strain exhibited resistance to penicillin, and one *Hafnia alvei* strain was resistant to both ampicillin and cefoxitin. Finally, all *Leuconostoc spp.* strains were resistant to vancomycin.

Determination of Antibiotic Resistance by Genotypic Methods

The expected sizes of the amplified fragments for each gene are reported in Table 4 (Appendix 1). Multiplex PCR assays performed on control strains showed the expected bands without nonspecific amplification. The results are summarized in Tables 21 (food matrices) and 22 (bacterial strains). In general, at least one antibiotic resistance gene was detected in all food matrices analyzed. In 7% of cases, only one resistance gene was present, whereas in 93% of cases, between two and eight resistance genes were detected simultaneously. The most frequently identified genes were those conferring resistance to tetracyclines (96%), followed by genes associated with erythromycin (66%), β-lactams (55%), and quinolones (38%). Regarding the bacterial strains, at least one antibiotic resistance gene was detected in 20 out of 58 strains (34.5%). Among these, four strains harbored two resistance genes and one strain carried three resistance genes. The genes most frequently associated with acquired tetracycline resistance were again the most prevalent (80%), followed by those related to β-lactams (25%), erythromycin (20%), and quinolones (5%).

Whole Genome Sequencing (WGS)

A total of 13 lactic acid bacteria strains isolated during the project were sequenced: seven *Lactococcus lactis* strains, two *Lactobacillus plantarum* strains, two *Lactococcus garvieae* strains, one

Leuconostoc mesenteroides strain, and one *Pediococcus pentosaceus* strain. The results concerning species identification and antibiotic resistance gene detection are summarized in Table 23 (Appendix 1). In both *Lactococcus garvieae* strains, genes associated with resistance to the following antibiotic classes were identified: aminoglycosides, lincosamides, macrolides, streptogramin B, and amphenicols. All other strains analyzed tested negative for antibiotic resistance genes.

Extraction, Antibacterial Activity, and Characterization of Bacteriocins

The results obtained from the in vitro evaluation of the antibacterial activity of bacteriocins are summarized in Table 24 (Appendix 1).

E. coli

A 50% MIC value was observed for *E. coli* in 13 analyzed cell-free supernatants (CFS). Most of these samples (11) were derived from bacteria belonging to the genus *Lactobacillus spp.*, while the remaining two originated from *Leuconostoc pseudomesenteroides* and *Streptococcus salivarius* subsp. *thermophilus*. In 4 out of the 13 samples, the minimum bactericidal concentration (MBC) coincided with the MIC, suggesting that the bacteriocin not only inhibited bacterial growth but also exerted a bactericidal effect. In an additional 23 samples, the MIC corresponded to the undiluted (100%) CFS; however, only in two of these cases did the MBC show the same value. Finally, in four samples, bacterial growth was observed even in the undiluted bacteriocin solution, suggesting lower efficacy of the bacteriocins under those conditions.

S. aureus

With regard to *S. aureus*, a 50% MIC value was detected in 11 bacteriocin samples extracted from lactic acid bacteria belonging to the genus *Lactobacillus spp.* Among these, four samples exhibited an MBC equal to the MIC, indicating a bactericidal effect. In 26 samples obtained from different genera (*Lactococcus spp.*—17, *Leuconostoc spp.*—5, *Streptococcus spp.*—3, and *Lactobacillus brevis*—1), bacterial growth was observed on the plates even with undiluted bacteriocin, indicating a bacteriostatic but not bactericidal effect against *S. aureus*.

In 11 bacteriocin samples derived from *Lactobacillus spp.*, a 50% MIC was detected against both *E. coli* and *S. aureus*. However, only one sample showed an MBC equal to the MIC for both bacteria, demonstrating full efficacy of the bacteriocin as both a growth inhibitor and a bactericidal agent.

Heat Resistance Tests

The results of the heat resistance assays conducted at 40 °C, 70 °C, and 100 °C, summarized in Tables 25, 26, and 27 (Appendix 1), revealed similar behavior against both *E. coli* and *S. aureus*. The CFS extracted from strain 26 (*Lactobacillus paracasei*) showed reduced bactericidal activity (MBC) after treatment at 40 °C, with a more pronounced decrease following exposure to 100 °C. Nevertheless, the MIC values for this strain were not affected by any of the heat treatments. The CFS obtained from strain 30 (*Lactobacillus plantarum*) exhibited reduced efficacy in terms of MIC only after exposure to 100 °C. For the CFS extracted from strain 58 (*Lactobacillus curvatus*), a progressive decrease in both MIC and MBC efficacy was observed starting from the 70 °C treatment. At 100 °C, the bactericidal effect of this bacteriocin was completely lost.

Evaluation of the Effect of pH

The results of the pH variation tests showed a similar trend for both pathogens, E. coli and S. aureus, consistent with the observations from the heat resistance assays. The MIC and MBC values at pH 3, 6, and 9 are reported in Tables 28, 29, and 30 (Appendix 1), respectively. The tests conducted at pH 3 revealed a reduction in bactericidal efficacy (MBC) for the CFS extracted from strain 26 (Lactobacillus paracasei) and a reduction in inhibitory efficacy (MIC) for the CFS from strain 58 (Lactobacillus curvatus). At pH 6, a complete loss of efficacy was observed for the CFS derived from strains 26 (Lactobacillus paracasei) and 30 (Lactobacillus plantarum). For the CFS extracted from strain 58 (Lactobacillus curvatus), the inhibitory effect (MIC) was maintained, but the bactericidal activity (MBC) was lost. At pH 9, none of the bacteriocins tested exhibited antibacterial activity.

Modulation of Innate Immunity

The bacterial strains used in this study showed differential modulation of genes involved in innate immune responses:

- Strain 30 (Lactobacillus plantarum) significantly reduced the gene expression of NFκB/p65 ($p = 0.02$), TLR4, TLR5, cGAS ($p = 0.01$), and IFNβ ($p = 0.04$).
- Strain 26 (Lactobacillus paracasei) significantly decreased the expression of TGFβ1 ($p = 0.03$).
- Strain 58 (Lactobacillus curvatus) significantly downregulated NFκB/p65 ($p = 0.03$) and IRF3 ($p = 0.047$), while upregulating CXCL8 and IL18 ($p = 0.01$) and TGFβ ($p = 0.05$).
- Strain 51 (Lacticaseibacillus paracasei) increased the expression of CXCL8 ($p = 0.04$).

Invasiveness

The strain used for this evaluation (Lactobacillus curvatus 58) did not inhibit the invasion of Salmonella typhimurium into IPEC-J2 cells. On the contrary, it facilitated bacterial entry, with a statistically significant increase ($p = 0.02$). These data confirm the association between the upregulation of pro-inflammatory molecules such as CXCL8 and the enhanced ability of Salmonella to invade porcine enterocytes.

Analysis by 16S rRNA Metagenomic Approach

The results of the two sampling sessions, divided by dairy farm, are shown in Figures 3 and 4. Each sample was analyzed in triplicate, and all replicates yielded overlapping results. Therefore, for each sample, the replicate with the highest total percentage of bacterial DNA validated by the analysis software was considered for downstream evaluation. In each pie chart, taxa with a relative abundance below 3.5% are grouped under the category "other." The microbiota of dairy products is influenced by numerous factors, including the health status of the animals, their diet, the season in which the milk is collected, and the methods used for milk storage. In all milk samples analyzed—regardless of their origin—a predominance of Gram-negative genera such as *Pseudomonas*, *Acinetobacter*, and *Chryseobacterium* was observed. As reported in previous studies, these genera tend to emerge and become dominant when milk is refrigerated prior to processing. Regarding the curd, a greater differentiation was observed. In dairy 2, the most represented genera were *Streptococcus*, *Acinetobacter*, and *Pseudomonas*, while in dairy 3, *Acinetobacter*, *Enterobacter*, and *Flavobacterium* were predominant. In the final cheese products, the following compositions were observed: for dairy 2, *Streptococcus*, *Leuconostoc*, and *Psychrobacter* dominated the summer production, whereas in the autumn production, *Psychrobacter* was replaced by *Enterobacter*. In dairy 3, the most represented genera were *Lactococcus* and *Serratia*, and similarly, *Enterobacter* appeared in the autumn samples. In parallel, the production chain was also monitored by characterizing the microbiota composition of the dairy environment through sponge sampling in three different production facilities: the two traditional Piedmontese dairies (2 and 3) and an experimental teaching dairy. Specifically, two different cheese types were analyzed by sampling consecutive stages of the production process. Samples collected and processed included raw milk, curd, cheese at mid- and end-ripening, and environmental swabs taken from the milk vats, the boiler, and the ripening boards.

Across all milk samples analyzed—regardless of origin—a predominance of *Pseudomonas*, *Acinetobacter*, and *Chryseobacterium* was confirmed. During the curd stage, a greater differentiation became evident: in Dairy A, the most abundant genera were *Streptococcus*, *Acinetobacter*, and *Pseudomonas*, while in Dairy B, *Acinetobacter*, *Enterobacter*, and *Flavobacterium* prevailed. Finally, regarding the cheese microbiota, the final product from Dairy A was dominated by *Streptococcus*, *Leuconostoc*, *Psychrobacter*, and *Enterobacter*. The cheese from Dairy B was characterized by *Lactococcus* and *Serratia* as the most abundant genera; again, *Enterobacter* appeared in the autumn production (Figures 5 and 6).

Statistical comparisons between sampling groups

Statistical analyses were applied to evaluate differences among predefined sampling groups, as described in the Materials and Methods section. Microbiological counts were compared between matrices (milk, curd and cheese). A statistically significant increase in total aerobic mesophilic bacteria and lactic acid bacteria counts was observed in cheese samples compared to milk and curd ($p < 0.05$), consistent with the progression of the fermentation process. When samples were grouped according to animal species, products derived from cow's milk showed significantly higher counts of Lactococcus spp., whereas goat milk products were characterized by a greater relative abundance of Lactobacillus spp. ($p < 0.05$). The distribution of antibiotic resistance phenotypes and genotypes among lactic acid bacteria was compared across genera and sampling matrices. No statistically significant association was observed between sampling matrix and the presence of acquired antibiotic resistance genes ($p > 0.05$), suggesting that resistance determinants were not specifically linked to a single production stage. Comparative analysis of bacteriocin activity among genera showed that cell-free supernatants produced by Lactobacillus spp. exhibited significantly lower MIC values against both Escherichia coli and Staphylococcus aureus compared to those produced by other lactic acid bacteria genera ($p < 0.05$). For RT-qPCR experiments, statistically significant modulation of innate immunity-related genes was confirmed for individual strains compared to untreated controls ($p < 0.05$), supporting the strain-specific immunomodulatory effects described above. Overall, statistical comparisons supported the descriptive findings, confirming that observed differences among sampling groups were not attributable to random variation.

CHAPTER V - DISCUSSION AND CONCLUSIONS

5.1 Food safety and hygiene of traditional raw milk cheeses

The first objective of this study was to evaluate the microbiological safety and hygiene status of traditional raw milk dairy products produced without industrial starter cultures. Overall, the results demonstrate that the analyzed products complied with European food safety criteria, as neither Salmonella spp. nor Listeria monocytogenes were detected in any cheese samples. Similar findings have been reported for artisanal raw milk cheeses when good manufacturing practices are applied, despite the absence of pasteurization (Cocolin et al., 2015; Parente et al., 2020). Process hygiene indicators showed greater variability. While most samples were classified as satisfactory or acceptable for Escherichia coli β-glucuronidase and coagulase-positive staphylococci, a limited number exceeded recommended thresholds. This variability has been frequently described in small-scale dairies and is generally attributed to differences in milking hygiene, equipment sanitation, and manual handling during cheesemaking (Ledenbach and Marshall, 2009; Quigley et al., 2013). Nevertheless, the low prevalence of unsatisfactory samples suggests that traditional cheesemaking practices do not inherently compromise food safety. The near-complete absence of antibiotic residues in milk and cheese samples further supports the safety of the investigated products. Comparable results have been reported in other studies on artisanal dairy productions, highlighting increased awareness of antimicrobial stewardship among small-scale farmers (Mitchell et al., 2012; Landers et al., 2012).

5.2 Microbiota composition and influence of production factors

A major aim of this work was to characterize the microbiota of traditional dairy products and to assess the influence of production-related factors. Both culture-dependent and NGS-based analyses revealed clear differences among milk, curd, and cheese matrices. Raw milk samples were dominated by psychrotrophic Gram-negative genera such as Pseudomonas, Acinetobacter, and Chryseobacterium. This microbial profile is well documented and is strongly associated with refrigerated storage prior to processing, which favors the growth of cold-tolerant bacteria (De Jonghe et al., 2011; Zotta et al., 2021). During curd formation and cheese ripening, a progressive shift toward lactic acid bacteria was observed, with Lactococcus, Lactobacillus, Leuconostoc, and Streptococcus emerging as dominant genera. These taxa are known to drive fermentation, acidification, and flavor development in raw milk cheeses (Fox et al., 2017; Parente et al., 2020). The higher similarity observed among cheeses produced within the same dairy compared to those produced in different facilities confirms the strong influence of the production environment, often referred to as the “house microbiota” (Bokulich and Mills, 2013; Wolfe et al., 2014). Seasonal variations, particularly the increased presence of Enterobacter in autumn samples, have also been reported in previous studies and may reflect changes in animal feeding, environmental conditions, and microbial loads in the farm environment (Dugat-Bony et al., 2016).

5.3 Antibiotic resistance in LAB and food matrices

The investigation of antibiotic resistance (AR) addressed a critical food safety concern. Phenotypic analyses revealed that most LAB strains exhibited limited resistance profiles, largely consistent with intrinsic resistance mechanisms. The widespread resistance to vancomycin observed in Lactobacillus and Leuconostoc spp. is well documented and is attributed to the absence of the D-Ala-D-Ala target rather than to acquired resistance genes (Danielsen and Wind, 2003; Anisimova et al., 2019). The lack of standardized breakpoint values for LAB complicates resistance interpretation, as previously highlighted by EFSA and other authors (EFSA, 2012; Ammor et al., 2007). For this reason, phenotypic data were interpreted in combination with genotypic analyses and literature evidence. In several cases, phenotypic resistance correlated with the detection of corresponding resistance genes, such as ermB in erythromycin-resistant Lactococcus garvieae strains and tet genes in tetracycline-resistant isolates, as reported in similar studies (Flórez et al., 2008; de Oliveira et al., 2022). Genotypic analyses revealed a high prevalence of AR genes in food matrices, whereas only a minority of isolated LAB strains carried detectable resistance genes. This discrepancy suggests that LAB are not the main contributors to the resistome of these dairy products, in agreement with previous findings indicating that environmental and opportunistic bacteria play a more significant role (Doyle et al., 2013; Devirgiliis et al., 2011). Whole genome sequencing confirmed this trend, with most LAB strains lacking acquired resistance genes. The detection of multiple resistance determinants in Lactococcus garvieae strains is consistent with the opportunistic pathogenic nature of this species and has been previously reported in both clinical and food-related isolates (Vendrell et al., 2006; Morandi et al., 2017).

5.4 Antibacterial activity and technological relevance of bacteriocins

The evaluation of bacteriocin activity highlighted the technological potential of LAB isolated from traditional cheeses. Bacteriocins produced by Lactobacillus spp. showed stronger antibacterial activity than those produced by other LAB genera, particularly against Escherichia coli. Similar observations have been reported for bacteriocins such as plantaricins and curvacins (Mohankumar et al., 2016; Cotter et al., 2013). The observed variability between bacteriostatic and bactericidal effects confirms that bacteriocin activity is highly strain-dependent. Thermal and pH stability tests further demonstrated that bacteriocin efficacy is influenced by processing conditions, as previously described for several LAB-derived antimicrobial peptides (Papagianni and Anastasiadou, 2009; Alvarez-Sieiro et al., 2016). These findings suggest that bacteriocins from selected Lactobacillus strains could be exploited as natural preservatives, provided that their stability under industrial processing conditions is carefully evaluated.

5.5 Immunomodulatory properties and safety considerations

The immunomodulatory assays revealed marked strain-specific effects on innate immune responses. The downregulation of NFκB and TLR signaling pathways observed for Lactobacillus plantarum strain 30 is consistent with previous reports describing anti-inflammatory properties of this species (Rocha-Ramírez et al., 2017; Lebeer et al., 2018). In contrast, Lactobacillus curvatus strain 58 exhibited a mixed immunomodulatory profile, with increased expression of CXCL8 and IL18. Similar strain-dependent effects have been reported for other LAB and underline the complexity of host–microbe interactions (Małaczewska et al., 2021). The observed facilitation of Salmonella typhimurium invasion further highlights the importance of safety assessment, as immune modulation may inadvertently favor pathogen colonization (Schmidt et al., 2008). These results reinforce the concept that probiotic and functional properties cannot be generalized at the species level and must be evaluated on an individual strain basis (Sanders et al., 2010).

5.6 Statistical considerations and data interpretation

The use of predefined sampling groups enabled comparative evaluations across matrices, animal species, production stages and bacterial genera, partially overcoming the intrinsic limitations related to sample size and heterogeneity. Statistical comparisons confirmed that the main trends observed in the microbiological and functional analyses were not attributable to random variation. In particular, the significant increase in lactic acid bacteria and total microbial counts from milk to cheese supported the expected dynamics of spontaneous fermentation in raw milk cheeses. Similarly, differences in the distribution of LAB genera between cow's and goat's milk products were statistically supported, reflecting the influence of raw material on microbial community structure. With regard to antibiotic resistance, the lack of statistically significant associations between sampling matrix and the presence of acquired resistance genes suggests that resistance determinants are not specifically linked to a single production stage. This finding supports the hypothesis that the resistome detected in the analyzed products is largely attributable to the broader microbial ecosystem rather than to the lactic acid bacteria population alone. This observation is consistent with previous studies indicating that LAB generally play a marginal role as reservoirs of transferable antibiotic resistance genes in fermented foods. Statistical analysis also supported the functional characterization of bacteriocins, confirming that bacteriocin activity was significantly associated with the producing genus. In particular, Lactobacillus-derived bacteriocins showed greater antibacterial efficacy compared to those produced by other LAB genera, reinforcing their potential application as natural antimicrobial agents in dairy products. Finally, statistical evaluation of RT-qPCR data confirmed the strain-specific nature of immunomodulatory effects, highlighting that immune modulation cannot be generalized at the species or genus level. These results underline the importance of individual strain characterization when considering the potential application of LAB or their metabolites in functional foods or probiotic formulations.

5.7 Conclusions

In conclusion, the results demonstrate that traditional raw milk cheeses can be microbiologically safe while hosting complex and product-specific microbial communities. LAB isolated from these products generally exhibit limited and mostly intrinsic antibiotic resistance, whereas resistance genes detected in food matrices are likely associated with non-LAB populations. Selected LAB strains produce bacteriocins with promising antibacterial activity, although their efficacy depends on environmental conditions. Finally, immunomodulatory effects are strongly strain-specific and must be carefully evaluated to ensure safety. Overall, this work contributes to the understanding of microbial ecology, safety, and functional potential of traditional dairy products and supports the preservation of artisanal cheesemaking practices within a scientifically sound safety framework.

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ANNEX 1 – TABLES

Table 1: Samples of cheese, curd, and milk collected

Samples number	Province	Producer	Sample Type	Sample description
1	Imperia	Caseificio 1	Formaggio	Toma Fresca
2		Caseificio 1	Latte	Latte vaccino
3	Biella	Caseificio 2	Latte	Latte vaccino
4		Caseificio 2	Cagliata	Cagliata
5		Caseificio 2	Primosale	Formaggio 1
6		Caseificio 2	Formaggio	Formaggio 2
7	Torino	Caseificio 3	Latte	Latte vaccino
8		Caseificio 3	Cagliata	Cagliata
9		Caseificio 3	Formaggio	Formaggio
10	Biella	Caseificio 4	Formaggio	Toma di capra
11		Caseificio 4	Latte	Latte caprino
12	Vercelli	Caseificio 5	Formaggio	Toma bovina
13		Caseificio 5	Latte	Latte vaccino
14		Caseificio 5	Formaggio	Toma di capra
15		Caseificio 5	Latte	Latte caprino
16	Biella	Caseificio 4	Formaggio	Toma bovina
17		Caseificio 4	Latte	Latte vaccino
18	Genova	Caseificio 6	Formaggio	Primo sale BV
19		Caseificio 6	Formaggio	Caciotta BV
20		Caseificio 6	Latte	Latte vaccino

21	Vercelli	Caseificio 7	Formaggio	Caprino valsesiano
22		Caseificio 7	Latte	Latte caprino
23		Caseificio 7	Formaggio	Toma Vaccina Valsesia
24		Caseificio 7	Latte	Latte vaccino
25	Vercelli	Caseificio 8	Formaggio	Toma Vaccina Valsesia
26		Caseificio 8	Latte	Latte vaccino
27		Caseificio 8	Formaggio	Caprino valsesiano
28		Caseificio 8	Latte	Latte caprino
29	Biella	Caseificio 2	Latte	Latte vaccino
30		Caseificio 2	Cagliata	Cagliata
31		Caseificio 2	Primosale	Formaggio 1
32		Caseificio 2	Formaggio	Formaggio 2
33	Torino	Caseificio 3	Latte	Latte vaccino
34		Caseificio 3	Cagliata	Cagliata
35		Caseificio 3	Primosale	Formaggio 1
36		Caseificio 3	Formaggio	Formaggio 2
37	Genova	Caseificio 6	Formaggio	Formaggio
38		Caseificio 6	Formaggio	Formaggio Tomino
39		Caseificio 6	Latte	Latte vaccino
40	Vercelli	Caseificio 9	Formaggio	Toma
41		Caseificio 9	Latte	Latte vaccino

Table 2: Molecules and their respective LOQs investigated using LC-HRMS in milk and cheese samples

Molecule	**U.M.**	**LOQ (ng/mL)**
Nalidixic acid	ng/mL	0,3
Florfenicol amine	ng/mL	0,3
Sulfadiazine	ng/mL	0,2
Sulfathiazole	ng/mL	0,2
Oxolinic acid	ng/mL	0,3
Flumequine	ng/mL	0,2
Sulfadimindine	ng/mL	0,2
Trimethoprim	ng/mL	0,2
Sulfadimethoxine	ng/mL	0,2
Cloramfenicol	ng/mL	0,1
Ciprofloxacin	ng/mL	0,2
Benzylpenicillin	ng/mL	0,6
Cephalexin	ng/mL	0,6
Ampicillin	ng/mL	0,6
Lomefloxacin	ng/mL	0,2
Thiamphenicol	ng/mL	0,2
Florfenicol	ng/mL	0,3
Enrofloxacin	ng/mL	0,2
Marbofloxacin	ng/mL	0,2
Amoxicillin	ng/mL	0,6
Lincomycin	ng/mL	0,2

Cloxacillin	ng/mL	0,7
Doxycycline	ng/mL	0,2
Tetracycline	ng/mL	0,2
Oxytetracycline	ng/mL	0,2
Dicloxacillin	ng/mL	0,7
Chlortetracycline	ng/mL	0,2
Cefquinome	ng/mL	0,6
Erythromycin	ng/mL	0,5
Tylosin	ng/mL	0,25
Tiamulina	ng/mL	0,25
Dimetridazolo	ng/mL	0,2
Ronidazolo	ng/mL	0,2
Tinidazolo	ng/mL	0,2
Sulfachloropyridazine	ng/mL	0,2
Sulfamethoxazole	ng/mL	0,2
Sulfamethoxypyridazine	ng/mL	0,2
Sulfamonomethoxin	ng/mL	0,2
Sulfapyridine	ng/mL	0,2
Sulfaquinoxaline	ng/mL	0,2
Tulathromicycin	ng/mL	0,5
Ceftiofur	ng/mL	0,3
Sulfamerazina	ng/mL	0,2
Danofloxacin	ng/mL	0,2
Tilmicosina	ng/mL	0,25

Spiramicina	ng/mL	0,5
Desfuroilceftiofur	ng/mL	0,3
Difloxacin	ng/mL	0,2
Norfloxacin	ng/mL	0,2
Levofloxacin-ofloxacin	ng/mL	0,2
Enoxacin	ng/mL	0,2
Cefadroxil	ng/mL	5
Cefalonio	ng/mL	5
Cefoperazone	ng/mL	5
Cefapirina	ng/mL	5
Cefazolina	ng/mL	5
Desacetilcefapirina	ng/mL	5
Cefalotina	ng/mL	5
Nafcillina	ng/mL	0,2
Oxacillina	ng/mL	0,2
Piperacillina	ng/mL	0,2
Oleandomicina	ng/mL	1
Josamicina	ng/mL	1
Valnemulina	ng/mL	1
Nitrofurantoina	ng/mL	0,1
Neospiramicina	ng/mL	1
Kitasamicina	ng/mL	1
Penicillina V	ng/mL	0,6
Nadifloxacin	ng/mL	0,2

Metronidazolo	ng/mL	0,2

Table 3: Sequences of primers of the target genes investigated

	Target genes	**Primer sequences (5’-3’)**
Multiplex PCR I	tet(B)	TTG GTT AGG GGC AAG TTT TG GTA ATG GGC CAA TAA CAC CG
Multiplex PCR II	tet(A)	GCT ACA TCC TGC TTG CCT TC CAT AGA TCG CCG TGA AGA GG
Multiplex PCR III	tet(K)	TCG ATA GGA ACA GCA GTA CAG CAG ATC CTA CTC CTT
	tet(L)	TCGTTA GCGTGC TGTCAT TC GTATCCCACCAATGTAGCCG
	tet(M)	GTGGACAAAGGT ACA ACGAG CGGTAAAGTTCGTCA CACAC
	tet(O)	AACTTAGGCATTCTGGCTCAC TCC CACTGTTCC ATATCGTCA
	tet(S)	CAT AGA CAA GCCGTT GACC ATG TTT TTG GAACGC CAG AG
Multiplex PCR IV	bla CTX-M	ATG TGCAGYACCAGTAARGTKATGGC TGG GTRAARTARGTSACCAGAAYCAGCGG
	bla TEM	CGCCGCATACACTATTCTCAGAATGA ACGCTCACCGGCTCCAGATTTAT
Multiplex PCR V	qnrA	ATTTCTCACGCCAGGATTTG

		GATCGGCAAAGGTTAGGTCA
	qnrB	GATCGTGAAAGCCAGAAAGG ACGATGCCTGGTAGTTGTCC
	qnrS	ACGACATTCGTCAACTGCAA TAAATTGGCACCCTGTAGGC
Multiplex PCR VI	ermA	GTTCAAGAACAATCAATACAGAG GGATCAGGAAAAGGACATTTTAC
	ermB	CCGTTTACGAAATTGGAACAGGTAAAGGGC GAATCGAGACTTGAGTGTGC
	ermC	GCTAATATTGTTTAAATCGTCAATTCC GGATCAGGAAAAGGACATTTTAC

Tabella 4: Annealing temperatures and amplicon sizes for each target gene

	Target genes	**Annealing temperature (°C)**	**Amplicons (bp)**
Multiplex PCRI	tet(B)	55°C	659
Multiplex PCRII	tet(A)	55°C	210
Multiplex PCRIII	tet(K)	55°C	169
	tet(L)	55°C	267
	tet(M)	55°C	406
	tet(O)	55°C	515
	tet(S)	55°C	667
Multiplex PCRIV	bla CTX-M	62°C	593
	bla TEM	62°C	445

Multiplex PCRV	qnr A	53°C	516
	qnr B	53°C	469
	qnr S	53°C	417
Multiplex PCRVI	erm A	53°C	421
	erm B	53°C	359
	erm C	53°C	572

Table 5: Primers used to assess gene expression modulation

Name	**Sequence**
IL6	F 5′-CAGAGATTTTGCCGAGGATG-3′
	R 5′-TGGCTACTGCCTTCCCTACC-3′
CXCL8	F 5′-CTGTACAACCTTCTGCACCCA-3′
	R 5′-TGGCTACTGCCTTCCCTACC-3′
IL18	F 5’-CGTGTTTGAGGATATGCCTGATT-3’
	R 5’-TGGTTACTGCCAGACCTCTAGTGA-3’
TNFα	F 5’-CTGGCCCAAGGACTCAGATCA-3’
	R 5’-CCAGGAGGGCATTGGCATAC-3’
TGFβ1	F 5’-AGCGCGATTTGCAGGTATTGA-3’
	R 5’-GCCGGTTGGACTGTTGTGAC-3’
NFkB/p65	F 5’-CGAGAGGAGCACGGATACCA-3’
	R 5’-GCCCCGTGTAGCCATTGA-3’
TLR4	F 5'-TGGCAGTTTCTGAGGAGTCATG-3’
	R 5'-CCGCAGCAGGGACTTCTC-3'

TLR5	F 5’-TCAAAGATCCTGACCATCACA-3’
	R 5’-CCAGCTGTATCAGGGAGCTT-3’
cGAS	F 5’-GGGTCCTGGGTACAGACGTG-3’
	R 5’-TGGAGTGAAATGTTGCAGGAAAGA-3’
IRF3	F 5’-GGGAAGGAGGCGTGTTCGAC-3’
	R 5’-ACCAGAGGGTGTAGCGTGGT-3’
IFNβ	F 5′-AGTTGCCTGGGACTCCTCAA-3′
	R 5′-CCTCAGGGACCTCAAAGTTCAT-3′
GAPDH	F 5’-ATGGTGAAGGTCGGAGTGAA-3’
	R 5’-AGTGGAGGTCAATGAAGGGG-3’

Table 6: Composition of the PCR mix for amplification of the 16S rRNA region

	Volume
Microbial DNA	2,5 µl
Amplicon PCR Forward 10 µM	1 µl
Amplicon PCR Reverse 10 µM	1 µl
Buffer	5 µl
PCRBIO HiFi Polymerase	0,25 µl
Water	15,25 µl
Tot.	25 µl

Table 7: Indices used as adapters for NGS sequencing

Index 1 (i7)	**Sequence**	**Index 2 (i5)**	**Sequence**
N701	TAAGGCGA	S501	TAGATCGC
N702	CGTACTAG	S502	CTCTCTAT
N703	AGGCAGAA	S503	TATCCTCT
N704	TCCTGAGC	S504	AGAGTAGA
N705	GGACTCCT	S505	GTAAGGAG
N706	TAGGCATG	S506	ACTGCATA
N707	CTCTCTAC	S507	AAGGAGTA
N708	CAGAGAGG	S508	CTAAGCCT
N709	GCTACGCT		
N710	CGAGGCTG		
N711	AAGAGGCA		
N712	GTAGAGGA		

Table 8: Results of microbiological analyses on cheeses

Samples	**Producer**	**Sample description**	***E. coli*** **ß glucoronidasi (UFC/g)**	**Enterobatteri (UFC/g)**	**Stafilococchi coagulasi positivi (UFC/g)**	**Carica mesofila a 30 °C (UFC/g)**	***Listeria monocytogenes***	***Salmonella* spp.**
Campione 1	Caseificio 1	Toma fresca	$1,5 \times 10^3$	$1,1 \times 10^3$	$7,7 \times 10^4$	$>3 \times 10^7$	Non rilevato	Non rilevato
Campione 6	Caseificio 2	Formaggio 2	$<1 \times 10^3$	$<1 \times 10^3$	$1,8 \times 10^4$	$3,1 \times 10^7$	Non rilevato	Non rilevato
Campione 9	Caseificio 3	Formaggio	$>1,5 \times 10^7$	$>1,5 \times 10^7$	$<1 \times 10^4$	$1,92 \times 10^9$	Non rilevato	Non rilevato
Campione 10	Caseificio 4	Toma di capra	<40	$1,2 \times 10^6$	70	$7,7 \times 10^8$	Non rilevato	Non rilevato
Campione 12	Caseificio 5	Toma Bovina	$4,1 \times 10^4$	2×10^6	<10	$7,8 \times 10^8$	Non rilevato	Non rilevato
Campione 14	Caseificio 5	Toma di capra	<10	1×10^7	<10	2×10^8	Non rilevato	Non rilevato
Campione 16	Caseificio 4	Toma bovina	$1,3 \times 10^2$	$2,5 \times 10^4$	$1,5 \times 10^2$	4×10^8	Non rilevato	Non rilevato
Campione 18	Caseificio 6	Primo sale BV	$2,9 \times 10^4$	$2,7 \times 10^7$	$1,2 \times 10^5$	$1,24 \times 10^8$	Non rilevato	Non rilevato
Campione 19	Caseificio 6	Caciotta BV	10	4×10^5	3×10^4	$7,06 \times 10^7$	Non rilevato	Non rilevato

Campione 21	Caseificio 7	Caprino valsesiano	2,9 x 10^4	1,2 x 10^7	2,8 x 10^4	3,5 x 10^8	Non rilevato	Non rilevato
Campione 23	Caseificio 7	Toma vaccina valsesia	5,5 x 10^4	6,5 x 10^5	<4 x 10^3	2,7 x 10^8	Non rilevato	Non rilevato
Campione 25	Caseificio 8	Toma Vaccina Valsesia	3,4 x 10^2	9,3 x 10^2	<10	8,7 x 10^8	Non rilevato	Non rilevato
Campione 27	Caseificio 8	Caprino valsesiano	1,6 x 10^3	7,7 x 10^3	<40	5,9 x 10^8	Non rilevato	Non rilevato
Campione 32	Caseificio 2	Formaggio 2	>10	1 x 10^6	>10	1,5 x 10^8	Non rilevato	Non rilevato
Campione 36	Caseificio 3	Formaggio 2	9,7 x 10^2	1,2 x 10^5	3,2 x 10^2	8,1 x 10^8	Non rilevato	Non rilevato
Campione 37	Caseificio 6	Formaggio	< 10	>3 x 10^4	1,2 x 10^3	2,4 x 10^8	Non rilevato	Non rilevato

Campione 38	Caseificio 6	Formaggio	1,7 x 10^{3}	>3 x 10^{4}	2,4 x 10^{5}	1,1 x 10^{9}	Non rilevato	Non rilevato
Campione 40	Caseificio 9	Toma	4 x 10^{4}	2,5 x 10^{4}	<10	2,8 x 10^{8}	Non rilevato	Non rilevato

Table 9: Results of Lactococci (M17) and Lactobacilli (MRS) counts at 30 °C and 37 °C

Samples	**Sample description**	**Lattobacilli 30°**	**Lattobacilli 37°**	**Lattococchi 30°**	**Lattococchi 37°**
Campione 1	Toma Fresca	970.000.000	230.000.000	150.000.000	260.000.000
Campione 3	Latte	1.020	470	72.000	12.400
Campione 4	Cagliata	204.000	740.000	64.000.000	350.000.000
Campione 5	Formaggio 1	<100000	<1000000	260.000.000	156.000.000
Campione 6	Formaggio 2	37.000.000	35.000.000	360.000.000	480.000.000
Campione 7	Latte	610.000	580.000	2.100.000	1.100.000
Campione 8	Cagliata	116.000.000	103.000.000	480.000.000	430.000.000

Campione 9	Formaggio	1.830.000.000	1.960.000.000	>1500000000	>1500000000
Campione 10	Toma di capra	460.000.000	250.000.000	370.000.000	260.000.000
Campione 12	Toma bovina	420.000.000	490.000.000	620.000.000	650.000.000
Campione 14	Toma di capra	420.000.000	500.000.000	610.000.000	430.000.000
Campione 16	Toma bovina	490.000.000	490.000.000	810.000.000	650.000.000
Campione 18	Primo sale BV	370.000.000	290.000.000	500.000.000	490.000.000
Campione 19	Caciotta BV	120.000.000	103.000.000	280.000.000	320.000.000
Campione 21	Caprino valsesiano	98.000.000	74.000.000	180.000.000	148.000.000
Campione 23	Toma Vaccina Valsesia	900.000.000	1.030.000.000	1.300.000.000	1.140.000.000
Campione 25	Toma Vaccina Valsesia	860.000.000	1.120.000.000	1.110.000.000	780.000.000
Campione 27	Caprino valsesiano	900.000.000	770.000.000	960.000.000	470.000.000
Campione 29	Latte	46.000	17.000	150.000	53.000
Campione 30	Cagliata	54.000	370.000	21.000.000	14.000.000
Campione 31	Formaggio 1	9.000.000	8.500.000	83.000.000	90.000.000
Campione 32	Formaggio 2	160.000.000	110.000.000	360.000.000	220.000.000
Campione 33	Latte	16.000	12.000	540.000	540.000

Campione 34	Cagliata	350.000	250.000	13.000.000	14.000.000
Campione 35	Formaggio 1	197.000.000	150.000.000	300.000.000	280.000.000
Campione 36	Formaggio 2	630.000.000	420.000.000	720.000.000	310.000.000
Campione 37	Formaggio	200.000.000	170.000.000	210.000.000	125.000.000
Campione 38	Formaggio	280.000.000	190.000.000	630.000.000	720.000.000
Campione 40	Toma	130.000.000	130.000.000	110.000.000	210.000.000

Table 10: results of bacterial colony identification using the MALDI-TOF system

Identification	**n. of strains**
Carnobacterium maltaromaticum	1
Citrobacter braakii	2
Enterobacter bugandensis	4
Enterobacter cloacae	1
Enterobacter kobei	2
Enterobacter ludwigii	1
Enterococcus durans	4
Enterococcus faecalis	20
Enterococcus faecium	5
Enterococcus gilvus	2
Hafnia alvei	7
Klebsialla oxytoca	4
Kluyveromyces marxianus (lievito)	1
Kocuria varians	4
Kurthia gibsonii	2
Lactobacillus paracasei	23
lactobacillus brevis	1
Lactobacillus curvatus	1
Lactobacillus delbrueckii	1
Lactobacillus fermentum	2
lactobacillus plantarum	8
Lactococcus garvieae	39

Lactococcus lactis	136
Lactococcus raffinolactis	2
Lelliottia amnigena	1
Leuconostoc mesenteroides	22
Leuconostoc pseudomesenteroides	4
Pediococcus pentosaceus	3
Rahnella aquatilis	1
Raoutella ornithinolytica	2
Serratia liquefaciens	2
Serratia marcescens	7
Staphylococcus aureus	3
Staphylococcus epidermidis	2
Staphylococcus hominis	1
Streptococcus agalactiae	1
Streptococcus equinus	2
Streptococcus gallolyticus	2
Streptococcus parauberis	2
Streptococcus salivarius ssp thermophilus	33
Streptococcus uberis	1

Table 11: Isolated lactic acid bacteria

N. strain	**Starting sample**	**Starting matrix**	**Species**	**Medium**	**Temp.**
1	Campione 1	Formaggio	*Lactococcus lactis*	MRS	30°C
2	Campione 1	Formaggio	*Lactococcus lactis*	M17	30°C
3	Campione 3	Latte	*Lactobacillus delbrueckii*	MRS	37°C
4	Campione 3	Latte	*Lactobacillus fermentum*	MRS	37°C
5	Campione 4	Cagliata	*Lactococcus lactis*	M17	37°C
6	Campione 4	Cagliata	*Streptococcus salivarius*_ssp_*thermophilus*	M17	37°C
7	Campione 5	Formaggio 1	*Streptococcus salivarius*_ssp_*thermophilus*	M17	37°C
8	Campione 5	Formaggio 1	*Streptococcus salivarius*_ssp_*thermophilus*	M17	30°C
9	Campione 6	Formaggio 2	*Staphylococcus hominis*	M17	30°C
10	Campione 6	Formaggio 2	*Leuconostoc mesenteroides*	MRS	30°C
11	Campione 7	Latte	*Lactococcus lactis*	MRS	37°C
12	Campione 8	Cagliata	*Lactococcus lactis*	M17	30°C
13	Campione 9	Formaggio	*Lactococcus lactis*	MRS	37°C
14	Campione 9	Formaggio	*Lactococcus lactis*	M17	37°C
15	Campione 10	Toma di capra	*Lactococcus lactis*	M17	37°C
16	Campione 10	Toma di capra	*Lactobacillus curvatus*	MRS	30°C

17	Campione 12	Toma bovina	*Lactococcus lactis*	M17	37°C
18	Campione 12	Toma bovina	*Lactobacillus paracasei*	MRS	30°C
19	Campione 14	Toma di capra	*Lactobacillus paracasei*	MRS	37°C
20	Campione 14	Toma di capra	*Lactococcus lactis*	M17	30°C
21	Campione 14	Toma di capra	*Leuconostoc mesenteroides*	MRS	30°C
22	Campione 16	Toma bovina	*Lactococcus lactis*	M17	37°C
23	Campione 16	Toma bovina	*Lactococcus lactis*	MRS	30°C
24	Campione 18	Primo sale	*Lactococcus garvieae*	MRS	37°C
25	Campione 18	Primo sale	*Enterococcus gilvus*	M17	30°C
26	Campione 19	Caciotta	*Lactobacillus paracasei*	MRS	30°C
27	Campione 19	Caciotta	*Lactobacillus plantarum*	MRS	37°C
28	Campione 21	Caprino	*Hafnia alvei*	MRS	30°C
29	Campione 23	Toma Vaccina	*Pediococcus pentosaceus*	MRS	30°C
30	Campione 25	Toma Vaccina	*Lactobacillus plantarum*	MRS	37°C

31	Campione 27	Caprino	*Lactobacillus brevis*	MRS	30°C
32	Campione 29	Latte	*Streptococcus equinus*	MRS	37°C
33	Campione 29	Latte	*Lactococcus lactis*	M17	37°C
34	Campione 29	Latte	*Lacticaseibacillus paracasei*	MRS	30°C
35	Campione 29	Latte	*Lactococcus raffinolactis*	M17	30°C
36	Campione 30	Cagliata	*Leuconostoc pseudomesenteroides*	MRS	37°C
37	Campione 30	Cagliata	*Leuconostoc mesenteroides*	MRS	37°C
38	Campione 30	Cagliata	*Streptococcus salivarius_ssp_thermophilus*	M17	37°C
39	Campione 30	Cagliata	*Leuconostoc mesenteroides*	MRS	30°C
40	Campione 30	Cagliata	*Lactococcus lactis*	M17	30°C
41	Campione 31	Primo sale	*Leuconostoc mesenteroides*	MRS	30°C
42	Campione 31	Primo sale	*Leuconostoc pseudomesenteroides*	MRS	37°C
43	Campione 31	Primo sale	*Streptococcus salivarius_ssp_thermophilus*	M17	37°C
44	Campione 32	Formaggio	*Leuconostoc mesenteroides*	MRS	30°C

45	Campione 32	Formaggio	*Streptococcus salivarius_ssp_thermophilus*	M17	30°C
46	Campione 33	Latte	*Lactococcus lactis*	MRS	30°C
47	Campione 34	Cagliata	*Lactococcus lactis*	MRS	30°C
48	Campione 35	Primo sale	*Lactococcus lactis*	M17	30°C
49	Campione 35	Primo sale	*Lactococcus lactis*	M17	37°C
50	Campione 36	Formaggio	*Lactobacillus plantarum*	MRS	30°C
51	Campione 36	Formaggio	*Lacticaseibacillus paracasei*	MRS	37°C
52	Campione 36	Formaggio	*Lactococcus lactis*	M17	37°C
53	Campione 37	Formaggio	*Lacticaseibacillus paracasei*	MRS	37°C
54	Campione 38	Formaggio	*Lactococcus garvieae*	MRS	37°C
55	Campione 38	Formaggio	*Lactococcus lactis*	M17	37°C
56	Campione 40	Formaggio	*Lactococcus lactis*	M17	30°C
57	Campione 40	Formaggio	*Lacticaseibacillus paracasei*	MRS	30°C
58	Campione 40	Formaggio	*Lactobacillus curvatus*	MRS	30°C

Table 12: Results of inhibitors in milk samples

N°	**sample**	**producer**	**species**	**CHARM**	**DELVO**	**Macrolidi**	**LC-HRMS**	**Molecule**	**ng/m**
1	Campione 2	Caseificio 1	Vaccino	Neg	Neg	Neg	Non eseguito	/	/
2	Campione 3	Caseificio 2	Vaccino	Neg	Neg	Neg	Neg	/	/
3	Campione 7	Caseificio 3	Vaccino	Neg	Neg	Neg	Neg	/	/
4	Campione 11	Caseificio 4	Caprino	Neg	Neg	Neg	Neg	/	/
5	Campione 17	Caseificio 4	Vaccino	Neg	Neg	Neg	Neg	/	/
6	Campione 13	Caseificio 5	Vaccino	Neg	Neg	Neg	Neg	/	/
7	Campione 15	Caseificio 5	Caprino	Neg	Neg	Neg	Neg	/	/
8	Campione 20	Caseificio 6	Vaccino	Neg	Neg	Neg	Neg	/	/
9	Campione 22	Caseificio 7	Vaccino	Neg	Neg	Neg	Neg	/	/
10	Campione 24	Caseificio 7	Caprino	Neg	Neg	Neg	Neg	/	/
11	Campione 26	Caseificio 8	Caprino	Neg	Neg	Neg	Neg	/	/
12	Campione 28	Caseificio 8	Vaccino	Neg	Neg	Neg	Neg	/	/
13	Campione 29	Caseificio 2	Vaccino	Neg	Neg	Neg	Neg	/	/
14	Campione 33	Caseifico 3	Vaccino	Neg	Neg	Neg	Neg	/	/

15	Campione 39	Caseificio 6	Vaccino	Neg	Neg	Neg	Neg	/	/
16	Campione 41	Caseificio 9	Vaccino	Neg	Neg	Neg	Neg	/	/

Table 13: Results of inhibitors in cheese samples at the end of ripening

N°	**sample**	**producer**	**species**	**LC-HRMS**	**Molecule**	**ng/m**
1	Campione 1	Caseificio 1	Vaccino	Non eseguito	/	/
2	Campione 6	Caseificio 2	Vaccino	Neg	/	/
3	Campione 9	Caseificio 3	Vaccino	Neg	/	/
4	Campione 10	Caseificio 4	Capra	Neg	/	/
5	Campione 16	Caseificio 4	Vaccino	Neg	/	/
6	Campione 19	Caseificio 6	Vaccino	Neg	/	/

7	Campione 12	Caseificio 5	Vaccino	Neg	/	/
8	Campione 14	Caseificio 5	Capra	Neg	/	/
9	Campione 18	Caseificio 6	Vaccino	Neg	/	/
10	Campione 23	Caseificio 7	Vaccino	Non eseguito	/	/
11	Campione 21	Caseificio 7	Capra	Neg	/	/
12	Campione 27	Caseificio 8	Capra	Neg	/	/
13	Campione 25	Caseificio 8	Vaccino	Neg	/	/
14	Campione	Caseificio 2	Vaccino	Neg	/	/
15	Campione	Caseificio 3	Vaccino	Neg	/	/
16	Campione	Caseificio 6	Vaccino	Neg	/	/
17	Campione	Caseificio 6	Vaccino	Neg	/	/

18	Campione	Caseificio 9	Vaccino	Pos	Lincomicina	3,65

Table 14: Diameter values (mm) obtained from Kirby-Bauer analysis for each strain tested

Num strain	**Species**	**Tetraciclina**	**Gentamicina**	**Eritromicina**	**Ciprofloxacina**	**Ampicillina**	**Streptomicina**
1	*Lactococcus lactis*	28	19	27	22	30	15
2	*Lactococcus lactis*	10	18	29	14	28	13
3	*Lactobacillus delbrueckii*	>30	19	>30	15	>30	25
4	*Lactobacillus fermentum*	19	25	25	13	>30	21
5	*Lactococcus lactis*	25	19	29	12	27	18
6	*Streptococcus salivarius* ssp. *thermophilus*	25	14	>30	21	>30	16
7	*Streptococcus salivarius* ssp. *thermophilus*	>30	15	>30	27	>30	25
8	*Streptococcus salivarius ssp. thermophilus*	25	16	23	20	26	14

9	*Staphylococcus hominis*	32	25	26	30	14	19
10	*Leuconostoc mesenteroides*	>30	>30	>30	27	>30	17
11	*Lactococcus lactis*	27	17	27	12	31	11
12	*Lactococcus lactis*	28	17	24	13	30	9
13	*Lactococcus lactis*	28	18	28	11	30	10
14	*Lactococcus lactis*	28	17	27	13	29	9
15	*Lactococcus lactis*	30	15	26	16	27	9
16	*Lactobacillus curvatus*	>30	16	>30	20	>30	17
17	*Lactococcus lactis*	26	16	24	16	>30	13
18	*Lactobacillus paracasei*	29	19	>30	26	>30	16
19	*Lactobacillus paracasei*	>30	19	>30	19	27	13
20	*Lactococcus lactis*	19	18	22	16	25	12
21	*Leuconostoc mesenteroides*	26	>30	>30	20	>30	>30
22	*Lactococcus lactis*	25	22	30	20	>30	14
23	*Lactococcus lactis*	25	20	14	20	>30	16
24	*Lactococcus garvieae*	24	27	<5	19	>30	10

25	*Enterococcus gilvus*	28	20	21	>30	>30	<5
26	*Lactobacillus paracasei*	28	22	>30	23	>30	10
27	*Lactobacillus plantarum*	<5	>30	>30	>30	23	18
28	*Hafnia alvei*	16	27	12	>30	19	22
29	*Pediococcus pentosaceus*	\	\	\	\	\	\
30	*Lactobacillus plantarum*	10	>30	>30	10	10	19
31	*Lactobacillus brevis*	>30	>30	>30	11	>30	>30
32	*Streptococcus equinus*	25	12	30	10	>30	<5
33	*Lactococcus lactis*	21	22	30	18	>30	16
34	*Lacticaseibacillus paracasei*	>30	21	>30	30	>30	10
35	*Lactococcus raffinolactis*	26	15	26	21	30	10
36	*Leuconostoc pseudomesenteroides*	>30	>30	>30	>30	>30	>30
37	*Leuconostoc mesenteroides*	29	30	>30	>30	>30	19
38	*Streptococcus salivarius ssp. thermophilus*	29	>30	>30	>30	>30	20
39	*Leuconostoc mesenteroides*	28	30	>30	>30	>30	20

40	*Lactococcus lactis*	24	25	30	16	>30	19
41	*Leuconostoc mesenteroides*	>30	>30	>30	>30	>30	18
42	*Leuconostoc pseudomesenteroides*	>30	>30	>30	>30	>30	>30
43	*Streptococcus salivarius_ssp_thermophilus*	>30	>30	>30	>30	>30	>30
44	*Leuconostoc mesenteroides*	28	>30	>30	26	>30	16
45	*Streptococcus salivarius ssp thermophilus*	>30	>30	>30	>30	>30	>30
46	*Lactococcus lactis*	21	22	30	20	30	19
47	*Lactococcus lactis*	28	26	>30	18	>30	16
48	*Lactococcus lactis*	26	21	30	20	30	16
49	*Lactococcus lactis*	26	21	30	19	30	14
50	*Lactobacillus plantarum*	<5	>30	>30	14	25	26
51	*Lacticaseibacillus paracasei*	29	25	>30	30	>30	19
52	*Lactococcus lactis*	20	17	30	18	>30	15
53	*Lacticaseibacillus paracasei*	30	22	>30	30	>30	11

54	*Lactococcus garvieae*	19	17	<5	18	29	<5
55	*Lactococcus lactis*	23	21	13	14	>30	12
56	*Lactococcus lactis*	20	18	30	14	30	12
57	*Lacticaseibacillus paracasei*	>30	24	>30	26	>30	18
58	*Lactobacillus curvatus*	23	24	>30	22	>30	11

Table 15: Antibiotic resistance profiles of the 58 LAB strains analyzed based on MIC

N. strain	Bacterial species	**CHL**	**DAP**	**GEN**	**LZD**	**RIF**	**SXT**	**SYN**	**TET**	**ERY**	**OXA**	**AMP**	**PEN**	**VAN**	**LEVO**	**TGC**	**MXF**	**CLI**	**STR**	**CIP**	**NIT**	**FOXS**	**GEN**	**KAN**	**STR**	**NEO**	**TET**	**ERY**	**CLI**	**CHL**
30	*Lactobacillus plantarum*	≤2	≤0.5	≤2	2	2	≤0.5/9.5	≤0.5	>16	≤0.25	≤0.25	0.5	1	>32	4	0.5	1	≤0.5	≤1000	>2	≤32	>6	≤0.5	≤2	2	≤0.12	8	≤0.015	0.12	1
31	*Lactobacillus brevis*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	1	16	≤0.25	≤0.25	0.5	1	>32	4	0.06	0.5	≤0.5	≤1000	>2	≤32	>6	≤0.5	≤2	1	≤0.12	4	≤0.015	0.12	0.5
1	*Lactococcus lactis*	8	≤0.5	≤2	2	>4	>4/76	2	>16	≤0.25	1	0.25	0.5	≤0.25	0.5	0.12	≤0.25	≤0.5	≤1000	2	64	>6	≤0.5	4	16	1	0.25	0.03	0.06	2
2	*Lactococcus lactis*	8	1	≤2	2	>4	>4/76	2	>16	≤0.25	1	0.25	0.5	≤0.25	≤0.25	≤0.03	≤0.25	≤0.5	≤1000	2	≤32	>6	≤0.5	≤2	8	1	64	≤0.015	≤0.03	≤0.12
3	*Lactobacillus delbrueckii*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	0.25	≤0.06	≤0.25	2	≤0.03	≤0.25	≤0.5	≤1000	2	≤32	≤6	/	/	/	/	/	/	/	/
4	*Lactobacillus fermentum*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	>32	2	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	≤0.5	≤2	≤0.5	≤0.12	≤0.12	≤0.015	≤0.03	0.5
5	*Lactococcus lactis*	8	≤0.5	≤2	4	>4	>4/76	2	<2	≤0.25	≤0.25	≤0.12	0.5	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	>2	≤32	>6	≤0.5	≤2	1	≤0.12	≤0.12	≤0.015	≤0.03	0.25
11	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	≤2	≤0.25	1	0.25	0.5	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	>2	64	>6	≤0.5	≤2	4	0.5	0.25	0.03	0.06	1
12	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	≤2	≤0.25	2	0.25	0.5	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	>2	>64	>6	1	4	8	1	0.25	0.03	0.06	1
13	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	≤2	≤0.25	2	0.25	0.5	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	>2	>64	>6	≤0.5	4	16	1	0.25	0.03	0.06	1
14	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	≤2	≤0.25	2	0.25	0.5	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	>2	>64	>6	1	≤2	8	1	0.25	0.03	0.06	≤0.12
15	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	1	≤2	≤0.25	0.5	0.25	0.5	≤0.25	1	0.06	≤0.25	≤0.5	≤1000	2	>64	>6	≤0.5	≤2	8	1	≤0.12	0.03	≤0.03	1
16	*Lactobacillus curvatus*	≤2	≤0.5	≤2	≤1		>4/76	≤0.5	≤2	≤0.25	2	0.5	0.5	>32	4	≤0.03	≤0.25	≤0.5	≤1000	>2	64	>6	≤0.5	4	16	1	1	≤0.015	≤0.03	1
17	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	1	≤2	≤0.25	1	0.25	0.25	≤0.25	1	0.06	≤0.25	≤0.5	≤1000	2	>64	>6	≤0.5	≤2	8	0.25	0.25	0.03	≤0.03	1
18	*Lactobacillus paracasei*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	1	1	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	8	8	1	0.5	≤0.015	≤0.03	1
19	*Lactobacillus paracasei*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	1	0.5	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	1	16	8	1	0.5	≤0.015	≤0.03	1
20	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	1	≤2	≤0.25	1	0.25	0.25	≤0.25	0.5	0.06	≤0.25	≤0.5	≤1000	2	>64	>6	≤0.5	≤2	8	0.5	≤0.12	0.03	≤0.03	≤0.12

22	*Lactococcus lactis*	≤2	≤0.5	≤2	≤1	4	≤0.5/9.5	2	≤2	≤0.25	0.5	≤0.12	0.25	≤0.25	0.5	0.06	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	8	0.5	≤0.12	≤0.015	≤0.03	1
23	*Lactococcus lactis*	4	≤0.5	≤2	2	4	≤0.5/9.5	2	≤2	≤0.25	0.5	≤0.12	0.5	≤0.25	0.5	0.06	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	4	0.25	0.25	0.03	≤0.03	1
24	*Lactococcus garvieae*	>16	≤0.5	4	≤1	>4	4/76	>4	≤2	>4	>4	1	1	0.5	1	0.12	≤0.25	>2	≤1000	2	≤32	>6	≤0.5	16	128	16	0.25	>8	>16	32
26	*Lactobacillus plantarum*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	1	≤2	≤0.25	2	1	0.5	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	16	8	1	0.5	≤0.015	≤0.03	1
27	*Lactobacillus paracasei*	≤2	≤0.5	≤2	≤1	4	≤0.5/9.5	1	16	≤0.25	≤0.25	1	4	>32	4	0.5	1	≤0.5	≤1000	>2	≤32	>6	≤0.5	≤2	4	≤0.12	4	≤0.015	0.12	1
33	*Lactococcus lactis*	≤2	≤0.5	≤2	≤1	>4	≤0.5/9.5	2	≤2	≤0.25	≤0.25	≤0.12	0.25	≤0.25	0.5	0.12	≤0.25	≤0.5	≤1000	2	≤32	>6	≤0.5	≤2	4	0.5	≤0.12	0.03	≤0.03	1
34	*Lacticaseibacillus paracasei*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	1	0.5	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	4	4	1	0.25	≤0.015	≤0.03	≤0.12
35	*Lactococcus raffinolactis*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	0.25	0.5	0.5	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	4	16	2	≤0.12	≤0.015	≤0.03	1
40	*Lactococcus lactis*	≤2	≤0.5	≤2	≤1	4	≤0.5/9.5	2	<2	≤0.25	≤0.25	≤0.12	0.25	≤0.25	0.5	0.06	≤0.25	≤0.5	≤1000	2	≤32	>6	≤0.5	≤2	4	0.25	≤0.12	≤0.015	≤0.03	1
46	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	<2	≤0.25	0.5	≤0.12	0.25	≤0.25	0.5	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	4	0.5	0.25	0.03	≤0.03	0.25
47	*Lactococcus lactis*	4	≤0.5	≤2	≤1	>4	≤0.5/9.5	≤0.5	<2	≤0.25	1	0.25	0.5	0.5	0.5	≤0.03	≤0.25	≤0.5	≤1000	2	≤32	>6	≤0.5	≤2	8	1	≤0.12	≤0.015	≤0.03	1
48	*Lactococcus lactis*	≤2	≤0.5	≤2	≤1	>4	≤0.5/9.5	2	<2	≤0.25	0.5	≤0.12	0.25	≤0.25	0.5	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	4	0.25	≤0.12	≤0.015	≤0.03	1
849	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	<2	≤0.25	0.5	≤0.12	0.25	≤0.25	0.5	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	16	0.5	≤0.12	0.03	≤0.03	1
50	*Lactobacillus plantarum*	4	≤0.5	≤2	2	2	≤0.5/9.5	1	>16	≤0.25	4	1	2	>32	4	0.12	0.5	≤0.5	≤1000	>2	≤32	>6	≤0.5	≤2	1	≤0.12	4	≤0.015	0.12	1
51	*Lacticaseibacillus paracasei*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	1	≤2	≤0.25	1	1	0.5	>32	1	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	8	4	2	0.5	≤0.015	≤0.03	0.5
52	*Lactococcus lactis*	≤2	≤0.5	≤2	≤1	>4	≤0.5/9.5	1	≤2	≤0.25	0.5	≤0.12	0.25	≤0.25	0.5	0.25	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	≤2	8	0.5	0.25	≤0.015	≤0.03	1
53	*Lacticaseibacillus paracasei*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	1	≤2	≤0.25	2	1	0.5	>32	1	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	16	8	2	0.5	≤0.015	≤0.03	1
54	*Lactococcus garvieae*	>16	≤0.5	4	2	>4	4/76	4	<2	>4	>4	1	1	0.5	1	0.25	≤0.25	>2	≤1000	≤1	≤32	>6	1	8	4	32	0.25	8	0.06	≤0.12
55	*Lactococcus lactis*	4	≤0.5	≤2	2	>4	≤0.5/9.5	2	<2	≤0.25	1	0.25	0.5	≤0.25	1	≤0.03	≤0.25	≤0.5	≤1000	>2	>64	>6	≤0.5	≤2	4	≤0.12	≤0.12	≤0.015	≤0.03	1
6	*Streptococcus salivarius ssp thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	≤0.25	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	/	/	/	/	/	/	/	/

7	*Streptococcus salivarius_ssp_thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	≤0.25	0.5	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	/	/	/	/	/	/	/	/
8	*Streptococcus salivarius_ssp_thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	≤0.25	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	/	/	/	/	/	/	/	/
9	*Staphylococcus hominis*	4	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	0.5	0.5	0.5	≤0.25	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	/	/	/	/	/	/	/	/
10	*Leuconostoc mesenteroides*	≤2	≤0.5	4	2	1	1/19	≤0.5	≤2	≤0.25	≤0.25	1	0.25	>32	2	≤0.03	≤0.25	≤0.5	≤1000	≤1	64	>6	/	/	/	/	/	/	/	/
25	*Enterococcus gilvus*	4	≤0.5	4	2	1	1/19	≤0.5	≤2	0.5	>4	0.5	1	0.5	1	0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	/	/	/	/	/	/	/	/
28	*Hafnia alvei*	8	>4	≤2	>8	>4	≤0.5/9.5	>4	8	>4	>4	4	>8	>32	≤0.25	0.5	≤0.25	>2	≤1000	≤1	≤32	>6	/	/	/	/	/	/	/	/
29	*Pediococcus pentosaceus*	≤2	≤0.5	≤2	4	2	4/76	2	>16	≤0.25	≤0.25	2	2	>32	>4	0.25	2	≤0.5	≤1000	>2	≤32	>6	/	/	/	/	/	/	/	/
32	*Streptococcus equinus*	≤2	≤0.5	8	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	0.12	≤0.25	1	0.06	≤0.25	≤0.5	≤1000	2	≤32	≤6	/	/	/	/	/	/	/	/
36	*Leuconostoc pseudomesenteroides*	8	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	1	0.5	0.12	>32	1	0.06	≤0.25	≤0.5	≤1000	≤1	>64	>6	/	/	/	/	/	/	/	/
37	*Leuconostoc mesenteroides*	≤2	≤0.5	≤2	≤1	≤0.5	2/38	≤0.5	≤2	≤0.25	≤0.25	0.5	0.25	>32	1	0.06	≤0.25	≤0.5	≤1000	2	64	>6	/	/	/	/	/	/	/	/
38	*Streptococcus salivarius_ssp_thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	≤0.25	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	/	/	/	/	/	/	/	/
39	*Leuconostoc mesenteroides*	4	≤0.5	≤2	≤1	>4	1/19	2	≤2	≤0.25	≤0.25	0.5	0.25	>32	1	0.06	≤0.25	≤0.5	≤1000	2	>64	>6	/	/	/	/	/	/	/	/
41	*Leuconostoc mesenteroides*	4	≤0.5	≤2	≤1	1	2/38	≤0.5	≤2	≤0.25	≤0.25	0.5	0.25	>32	2	≤0.03	≤0.25	≤0.5	≤1000	2	>64	>6	/	/	/	/	/	/	/	/
42	*Leuconostoc pseudomesenteroides*	4	≤0.5	≤2	2	1	1/19	1	≤2	≤0.25	1	0.5	0.25	>32	2	≤0.03	≤0.25	≤0.5	≤1000	2	>64	>6	/	/	/	/	/	/	/	/
43	*Streptococcus salivarius_ssp_thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	0.5	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	≤6	/	/	/	/	/	/	/	/
44	*Leuconostoc mesenteroides*	≤2	≤0.5	≤2	≤1	1	1/19	≤0.5	≤2	≤0.25	≤0.25	0.5	0.25	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	>64	≤6	/	/	/	/	/	/	/	/

45	*Streptococcus salivarius_ssp_thermophilus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	≤0.25	≤0.12	≤0.06	≤0.25	1	0.12	≤0.25	≤0.5	≤1000	≤1	≤32	>6	/	/	/	/	/	/	/	/
56	*Lactococcus lactis*	4	>4	≤2	2	>4	≤0.5/9.5	4	≤2	>4	2	0.25	0.5	≤0.25	1	0.06	≤0.25	>2	≤1000	>2	>64	>6	≤0.5	≤2	8	1	0.25	>8	>16	2
57	*Lacticaseibacillus paracasei*	4	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	1	≤2	≤0.25	2	2	1	>32	1	≤0.03	≤0.25	≤0.5	≤1000	≤1	≤32	>6	≤0.5	4	4	0.5	1	≤0.015	≤0.03	2
58	*Lactobacillus curvatus*	≤2	≤0.5	≤2	≤1	≤0.5	≤0.5/9.5	≤0.5	≤2	≤0.25	1	1	0.5	>32	4	≤0.03	1	≤0.5	≤1000	>2	≤32	>6	≤0.5	4	8	0.5	1	0.03	≤0.03	2

Table 16: Interpretation of MIC values obtained with reference to the genus *Lactobacillus* spp.

N. STRAIN	BACTERIAL SPECIES	DAP	LZD	ERY	AMP	PEN	VAN	CLI
3	*Lactobacillus delbrueckii*	S	S	S	S	S	S	S
4	*Lactobacillus fermentum*	S	S	S	S	S	R	S
16	*Lactobacillus curvatus*	S	S	S	S	S	R	S
18	*Lactobacillus paracasei*	S	S	S	S	S	R	S
19	*Lactobacillus paracasei*	S	S	S	S	S	R	S
26	*Lactobacillus paracasei*	S	S	S	S	S	R	S
27	*Lactobacillus plantarum*	S	S	S	S	S	R	S
30	*Lactobacillus plantarum*	S	S	S	S	S	R	S
31	*Lactobacillus brevis*	S	S	S	S	S	R	S
34	*Lacticaseibacillus paracasei*	S	S	S	S	S	R	S
50	*Lactobacillus plantarum*	S	S	S	S	S	R	S
51	*Lacticaseibacillus paracasei*	S	S	S	S	S	R	S
53	*Lacticaseibacillus paracasei*	S	S	S	S	S	R	S
57	*Lacticaseibacillus paracasei*	S	S	S	S	S	R	S
58	*Lactobacillus curvatus*	S	S	S	S	S	R	S

Table 17: Interpretation of MIC values obtained with reference to the genus *Lactococcus* spp.

N. STRAIN	**BACTERIAL SPECIES**	**SXT**	**TET**	**ERY**	**AMP**	**PEN**	**VAN**	**LEVO**	**CLI**
1	*Lactococcus lactis*	R	R	S	S	S	S	S	S
2	*Lactococcus lactis*	R	R	S	S	S	S	S	S
5	*Lactococcus lactis*	R	S	S	S	S	S	S	S
11	*Lactococcus lactis*	S	S	S	S	S	S	S	S
12	*Lactococcus lactis*	S	S	S	S	S	S	S	S
13	*Lactococcus lactis*	S	S	S	S	S	S	S	S
14	*Lactococcus lactis*	S	S	S	S	S	S	S	S
15	*Lactococcus lactis*	S	S	S	S	S	S	S	S
17	*Lactococcus lactis*	S	S	S	S	S	S	S	S
20	*Lactococcus lactis*	S	S	S	S	S	S	S	S
22	*Lactococcus lactis*	S	S	S	S	S	S	S	S
23	*Lactococcus lactis*	S	S	S	S	S	S	S	S
24	*Lactococcus garvieae*	R	S	R	S	S	S	S	R
33	*Lactococcus lactis*	S	S	S	S	S	S	S	S
35	*Lactococcus raffinolactis*	S	S	S	S	S	S	S	S
40	*Lactococcus lactis*	S	S	S	S	S	S	S	S
46	*Lactococcus lactis*	S	S	S	S	S	S	S	S
47	*Lactococcus lactis*	S	S	S	S	S	S	S	S
48	*Lactococcus lactis*	S	S	S	S	S	S	S	S

49	*Lactococcus lactis*	S	S	S	S	S	S	S	S
52	*Lactococcus lactis*	S	S	S	S	S	S	S	S
54	*Lactococcus garvieae*	R	S	R	S	S	S	S	S
55	*Lactococcus lactis*	S	S	S	S	S	S	S	S
56	*Lactococcus lactis*	S	S	R	S	S	S	S	R

Table 18: Interpretation of MIC values obtained with reference to the genus *Streptococcus* spp.

*MIC values compared with EUCAST breakpoints

N. Strain	**BACTERIAL SPECIES**	**CHL**	**DAP**	**GEN***	**LZD**	**RIF***	**SYN**	**TET**	**ERY**	**AMP**	**PEN**	**VAN**	**LEVO**	**MXF***	**CLI**
6	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
7	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
8	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S

32	*Streptococcus equinus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
38	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
43	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S
45	*Streptococcus salivarius* ssp *thermophilus*	S	S	S	S	S	S	S	S	S	S	S	S	S	S

Table 19: Interpretation of MIC values obtained with reference to the genus *Leuconostoc* spp.

Num. STRAIN	**BACTERIAL SPECIES**	**CHL**	**AMP**	**PEN**	**VAN**
10	*Leuconostoc mesenteroides*	S	S	S	R
21	*Leuconostoc mesenteroides*	S	S	S	R
36	*Leuconostoc pseudomesenteroides*	S	S	S	R
37	*Leuconostoc mesenteroides*	S	S	S	R
39	*Leuconostoc mesenteroides*	S	S	S	R

41	*Leuconostoc mesenteroides*	S	S	S	R
42	*Leuconostoc pseudomesenteroides*	S	S	S	R
44	*Leuconostoc mesenteroides*	S	S	S	R

Table 20: Interpretation of MIC values obtained with reference to the bacterial species *Staphylococcus hominis, Enterococcus gilvus, Hafnia alvei, Pediococcus pentosaceus*. MIC values compared with CLSI breakpoints

N. strain	**BACTERIAL SPECIES**	**CHL**	**DAP**	**GEN**	**LZD**	**RIF**	**SXT**	**SYN**	**TET**	**ERY**	**OXA**	**AMP**	**PEN**	**VAN**	**LEVO**	**TGC**	**MXF**	**CLI**	**CIP**	**NIT**	**FOXS**
9	*Staphylococcus hominis*	/	S	S	S	S	S	S	S *	S	S	/	R	S	I	S	S	S *	I	S	/
25	*Enterococcus gilvus*	/	/	S	S	/	S	S	/	/	/	S	/	S	S	S	S	/	S	S	/
28	*Hafnia alvei*	/	/	/	/	/	/	/	/	/	/	R	/	/	/	/	/	/	/	/	R
29	*Pediococcus pentosaceus*	S	/	/	/	/	/	/	/	/	/	S	S	/	/	/	/	/	/	/	/

Table 21: Results of the presence of antibiotic resistance genes in the tested food matrices.
tet = tetracycline resistance genes; erm = erythromycin resistance genes; bla = β-lactam resistance genes; qnr = quinolone resistance genes

Num. sample	**Producer**	**Product**	+
1	Caseificio 1	Toma Fresca	tet (M), tet (S)
2	Caseificio 2	Latte	erm (A)
3	Caseificio 2	Cagliata	tet (M)
4	Caseificio 2	Formaggio 1	tet (M), tet (S), erm (B)
5	Caseificio 2	Formaggio 2	tet (B), tet (M), erm (B)
6	Caseificio 3	Latte	tet (B), tet (M), bla CTX-M, bla TEM, erm (B)
7	Caseificio 3	Cagliata	tet (B), tet (M), tet (O), tet (S), bla CTX-M, bla TEM, qnr (B), erm (B)
8	Caseificio 3	Formaggio	tet (A), tet (M), bla CTX-M, erm (B), erm (C)
9	Caseificio 4	Toma di capra	tet (M), erm (B)
10	Caseificio 5	Toma bovina	tet (M), bla CTX-M
11	Caseificio 5	Toma di capra	tet (A), tet (M), tet (S), bla CTX-M
12	Caseificio 4	Toma bovina	tet (B), tet (A), tet (M), tet (S), bla CTX-M, qnr (B), erm (B), erm (C)
13	Caseificio 6	Primo sale BV	tet (M), tet (O), tet (S), bla CTX-M, erm (B)
14	Caseificio 6	Caciotta BV	tet (M), tet (O), bla CTX-M, erm (B)
15	Caseificio 7	Caprino valsesiano	tet (B), tet (O), tet (S), bla CTX-M, bla TEM, erm (B)
16	Caseificio 7	Toma Vaccina Valsesia	tet (A), tet (O), tet (S), erm (B)
17	Caseificio 8	Toma Vaccina Valsesia	tet (B), tet (A), tet (L), tet (M), tet (S), qnr (B), erm (B)
18	Caseificio 8	Caprino valsesiano	tet (L), tet (M), tet (S), bla CTX-M, qnr (B), erm (B)

19	Caseificio 2	Latte	tet (M), bla TEM, qnr (B), erm (B)
20	Caseificio 2	Cagliata	tet (B), tet (M), tet (S), qnr (B)
21	Caseificio 2	Primo sale	tet (M), tet (S), bla TEM, qnr (B)
22	Caseificio 2	Formaggio	tet (B), tet (M), tet (S), bla CTX-M
23	Caseificio 3	Latte	tet (K), tet (M), qnr (B), qnr (S)
24	Caseificio 3	Cagliata	tet (K), qnr (A), qnr (B), qnr (S)
25	Caseificio 3	Primo sale	tet (B), tet (M), bla CTX-M, qnr (A), qnr (B), qnr (S), erm(B)
26	Caseificio 3	Formaggio	tet (B), tet (M), bla CTX-M, erm(B)
27	Caseificio 6	Formaggio	t tet (B), et (M), tet (O), tet (S), erm(B)
28	Caseificio 6	Tomino	tet (M), tet (S), bla CTX-M, qnr (B), erm(B)
29	Caseificio 9	Toma	tet (L), tet (M), tet (S), erm(B)

Table 22: Results of the presence of antibiotic resistance genes in the isolated bacterial strains.
tet = genes conferring resistance to tetracyclines;
erm = genes conferring resistance to erythromycin;
bla = genes conferring resistance to β-lactams;
qnr = genes conferring resistance to quinolones.

n. strain	**Starting sample**	**Producer**	**Product**	**Species**	+
1	Campione 1	Caseificio 1	Formaggio	*Lactococcus lactis*	
2	Campione 1	Caseificio 1	Formaggio	*Lactococcus lactis*	
3	Campione 2	Caseificio 2	Latte	*Lactobacillus delbrueckii*	tet (M)

4	Campione 2	Caseificio 2	Latte	*Lactobacillus fermentum*	
5	Campione 3	Caseificio 2	Cagliata	*Lactococcus lactis*	tet (K)
6	Campione 4	Caseificio 2	Cagliata	*Streptococcus salivarius_ssp_thermophilus*	
7	Campione 5	Caseificio 2	Formaggio 1	*Streptococcus salivarius*_ssp_*thermophilus*	
8	Campione 5	Caseificio 2	Formaggio 1	*Streptococcus salivarius*_ssp_*thermophilus*	
9	Campione 6	Caseificio 2	Formaggio 2	*Staphylococcus hominis*	
10	Campione 6	Caseificio 2	Formaggio 2	*Leuconostoc mesenteroides*	tet (S)
11	Campione 7	Caseificio 3	Latte	*Lactococcus lactis*	
12	Campione 8	Caseificio 3	Cagliata	*Lactococcus lactis*	
13	Campione 9	Caseificio 3	Formaggio	*Lactococcus lactis*	
14	Campione 9	Caseificio 3	Formaggio	*Lactococcus lactis*	
15	Campione 10	Caseificio 4	Toma di capra	*Lactococcus lactis*	
16	Campione 10	Caseificio 4	Toma di capra	*Lactobacillus curvatus*	
17	Campione 12	Caseificio 5	Toma bovina	*Lactococcus lactis*	tet (K)
18	Campione 12	Caseificio 5	Toma bovina	*Lactobacillus paracasei*	
19	Campione 14	Caseificio 5	Toma di capra	*Lactobacillus paracasei*	
20	Campione 14	Caseificio 5	Toma di capra	*Lactococcus lactis*	tet (K)
21	Campione 14	Caseificio 5	Toma di capra	*Leuconostoc mesenteroides*	

22	Campione 16	Caseificio 4	Toma bovina	*Lactococcus lactis*	tet (K)
23	Campione 16	Caseificio 4	Toma bovina	*Lactococcus lactis*	tet (M)
24	Campione 18	Caseificio 6	Primo sale	*Lactococcus garvieae*	tet (B), erm (B)
25	Campione 18	Caseificio 6	Primo sale	*Enterococcus gilvus*	
26	Campione 19	Caseificio 6	Caciotta	*Lactobacillus paracasei*	
27	Campione 19	Caseificio 6	Caciotta	*Lactobacillus plantarum*	
28	Campione 21	Caseificio 7	Caprino	*Hafnia alvei*	
29	Campione 23	Caseificio 7	Toma Vaccina	*Pediococcus pentosaceus*	
30	Campione 25	Caseificio 8	Toma Vaccina	*Lactobacillus plantarum*	
31	Campione 27	Caseificio 8	Caprino	*Lactobacillus brevis*	
32	Campione 29	Caseificio 2	Latte	*Streptococcus equinus*	bla CTX-M, erm(B)
33	Campione 29	Caseificio 2	Latte	*Lactococcus lactis*	
34	Campione 29	Caseificio 2	Latte	*Lacticaseibacillus paracasei*	bla TEM
35	Campione 29	Caseificio 2	Latte	*Lactococcus raffinolactis*	
36	Campione 30	Caseificio 2	Cagliata	*Leuconostoc pseudomesenteroides*	tet (K)
37	Campione 30	Caseificio 2	Cagliata	*Leuconostoc mesenteroides*	qnr (S)
38	Campione 30	Caseificio 2	Cagliata	*Streptococcus salivarius_ssp_thermophilus*	tet (K), tet (L), bla CTX-M,
39	Campione 30	Caseificio 2	Cagliata	*Leuconostoc mesenteroides*	
40	Campione 30	Caseificio 2	Cagliata	*Lactococcus lactis*	
41	Campione 31	Caseificio 2	Primo sale	*Leuconostoc mesenteroides*	tet (K)

42	Campione 31	Caseificio 2	Primo sale	*Leuconostoc pseudomesenteroides*	tet (M)
43	Campione 31	Caseificio 2	Primo sale	*Streptococcus salivarius_ssp_thermophilus*	
44	Campione 32	Caseificio 2	Formaggio	*Leuconostoc mesenteroides*	
45	Campione 32	Caseificio 2	Formaggio	*Streptococcus salivarius_ssp_thermophilus*	
46	Campione 33	Caseificio 3	Latte	*Lactococcus lactis*	
47	Campione 34	Caseificio 3	Cagliata	*Lactococcus lactis*	
48	Campione 35	Caseificio 3	Primo sale	*Lactococcus lactis*	
49	Campione 35	Caseificio 3	Primo sale	*Lactococcus lactis*	
50	Campione 36	Caseificio 3	Formaggio	*Lactobacillus plantarum*	tet (K)
51	Campione 36	Caseificio 3	Formaggio	*Lacticaseibacillus paracasei*	
52	Campione 36	Caseificio 3	Formaggio	*Lactococcus lactis*	
53	Campione 37	Caseificio 6	Formaggio	*Lacticaseibacillus paracasei*	
54	Campione 38	Caseificio 6	Formaggio	*Lactococcus garvieae*	tet (K), erm (B)
55	Campione 38	Caseificio 6	Formaggio	*Lactococcus lactis*	tet (K), erm (A)
56	Campione 40	Caseificio 9	Formaggio	*Lactococcus lactis*	erm (B)
57	Campione 40	Caseificio 9	Formaggio	*Lacticaseibacillus paracasei*	bla TEM
58	Campione 40	Caseificio 9	Formaggio	*Lactobacillus curvatus*	bla TEM

Table 23. Results of species identification and of the search for resistance genes by WGS

Num strain	**Bacterial species (KmerFinder-3.2)**	**Predicted phenotype (ResFinder)**	**Antibiotic**	**Class**	**Genetic background**
54	*Lactococcus garvieae*	Resistente	streptomycin	aminoglycoside	str;;1;;X92946
			lincomycin	lincosamide	erm(B);;18;;X66468
			clindamycin	lincosamide	erm(B);;18;;X66468
			erythromycin	macrolide	erm(B);;18;;X66468
			quinupristin	streptogramin b	erm(B);;18;;X66468
			pristinamycin ia	streptogramin b	erm(B);;18;;X66468
			virginiamycin s	streptogramin b	erm(B);;18;;X66468
			chloramphenicol	amphenicol	cat;;5;;U35036
24	*Lactococcus garvieae*	Resistente	streptomycin	aminoglycoside	str (str_X92946)
			lincomycin	lincosamide	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)
			clindamycin	lincosamide	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)
			erythromycin	macrolide	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)

			quinupristin	streptogramin b	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)
			pristinamycin ia	streptogramin b	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)
			virginiamycin s	streptogramin b	erm(B) (erm(B)_JN899585), erm(B) (erm(B)_X66468)
			chloramphenicol	amphenicol	cat (cat_U35036)
17	*Lactococcus lactis*	Non resistente	/	/	/
20	*Lactococcus lactis*	Non resistente	/	/	/
23	*Lactococcus lactis*	Non resistente	/	/	/
27	*Lactobacillus plantarum*	Non resistente	/	/	/
30	*Lactobacillus plantarum*	Non resistente	/	/	/
21	*Leuconostoc mesenteroides*	Non resistente	/	/	/
11	*Lactococcus lactis*	Non resistente	/	/	/
15	*Lactococcus lactis*	Non resistente	/	/	/

56	*Lactococcus lactis*	Non resistente	/	/	/
46	*Lactococcus lactis*	Non resistente	/	/	/
29	*Pediococcus pentosaceus*	Non resistente	/	/	/

Table 24: Results of the in vitro evaluation of the antibacterial activity of bacteriocins

	N. strain	**Species**	**MIC**		**MBC**	
			E. coli	*S. aureus*	*E. coli*	*S. aureus*
1	**30**	*Lactobacillus plantarum*	50%	50%	100%	100%
2	**31**	*Lactobacillus brevis*	100%	100%	100%	/
3	**1**	*Lactococcus lactis*	100%	100%	/	/
4	**2**	*Lactococcus lactis*	100%	100%	/	/
5	**5**	*Lactococcus lactis*	100%	100%	/	/
6	**6**	*Streptococcus salivarius* ssp *thermophilus*	50%	100%	100%	/
7	**10**	*Leuconostoc mesenteroides*	/	100%	/	/
8	**11**	*Lactococcus lactis*	100%	100%	/	/
9	**15**	*Lactococcus lactis*	100%	100%	/	/
10	**17**	*Lactococcus lactis*	100%	100%	/	/
11	**18**	*Lactobacillus paracasei*	50%	50%	50%	100%
12	**19**	*Lactobacillus paracasei*	50%	50%	50%	100%
13	**20**	*Lactococcus lactis*	100%	100%	/	/

14	**21**	*Leuconostoc mesenteroides*	100%	100%	/	/
15	**23**	*Lactococcus lactis*	100%	100%	/	/
16	**24**	*Lactococcus garvieae*	100%	100%	/	/
17	**26**	*Lactobacillus paracasei*	50%	50%	100%	100%
18	**27**	*Lactobacillus plantarum*	50%	50%	50%	50%
19	**56**	*Lactococcus lactis*	100%	/	/	/
20	**57**	*Lacticaseibacillus paracasei*	50%	50%	100%	100%
21	**58**	*Lactobacillus curvatus*	50%	50%	100%	100%
22	**32**	*Streptococcus equinus*	100%	100%	/	/
23	**42**	*Leuconostoc pseudomesenteroides*	50%	100%	100%	/
24	**44**	*Leuconostoc mesenteroides*	100%	100%	/	/
25	**33**	*Lactococcus lactis*	100%	100%	/	/
26	**34**	*Lacticaseibacillus paracasei*	50%	50%	100%	100%
27	**36**	*Leuconostoc pseudomesenteroides*	100%	100%	/	/
28	**38**	*Streptococcus salivarius_ssp_thermophilus*	/	/	/	/
29	**46**	*Lactococcus lactis*	100%	100%	/	/
30	**47**	*Lactococcus lactis*	100%	100%	/	/
31	**48**	*Lactococcus lactis*	100%	100%	/	/
32	**49**	*Lactococcus lactis*	100%	100%	/	/
33	**50**	*Lactobacillus plantarum*	50%	50%	100%	100%
34	**52**	*Lactococcus lactis*	100%	100%	/	/
35	**53**	*Lacticaseibacillus paracasei*	50%	50%	100%	100%
36	**54**	*Lactococcus garvieae*	100%	100%	/	/

37	**55**	*Lactococcus lactis*	100%	100%	100%	/
38	**45**	*Streptococcus salivarius_ssp_thermophilus*	/	/	/	/
39	**43**	*Streptococcus salivarius_ssp_thermophilus*	/	100%	/	/
40	**51**	*Lacticaseibacillus paracasei*	50%	50%	50%	100%

Table 25: Results of the in vitro evaluation of the antibacterial activity of bacteriocins following heat treatment at 40 °C

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	50%	50%	100%	100%
30	*Lactobacillus plantarum*	50%	50%	100%	100%
58	*Lactobacillus curvatus*	50%	50%	100%	100%

Table 26: Results of the in vitro evaluation of the antibacterial activity of bacteriocins following heat treatment at 70 °C

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	50%	50%	100%	100%
30	*Lactobacillus plantarum*	50%	50%	100%	100%
58	*Lactobacillus curvatus*	100%	100%	100%	100%

Table 27: Results of the in vitro evaluation of the antibacterial activity of bacteriocins following heat treatment at 100 °C.

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	50%	50%	100%	100%
30	*Lactobacillus plantarum*	100%	100%	100%	100%
58	*Lactobacillus curvatus*	100%	100%	/	/

Table 28: Results of the in vitro evaluation of the antibacterial activity of bacteriocins at pH 3

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	50%	50%	100%	100%
30	*Lactobacillus plantarum*	50%	50%	100%	100%
58	*Lactobacillus curvatus*	100%	100%	100%	100%

Table 29: Results of the in vitro evaluation of the antibacterial activity of bacteriocins at pH 6

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	/	/	/	/
30	*Lactobacillus plantarum*	/	/	/	/
58	*Lactobacillus curvatus*	100%	100%	/	/

Table 30: Results of the in vitro evaluation of the antibacterial activity of bacteriocins at pH 9

		MIC		**MBC**	
N. strain	Species	*E. coli*	*S. aureus*	*E. coli*	*S. aureus*
26	*Lactobacillus paracasei*	/	/	/	/
30	*Lactobacillus plantarum*	/	/	/	/
58	*Lactobacillus curvatus*	/	/	/	/

ANNEX 2 – FIGURES

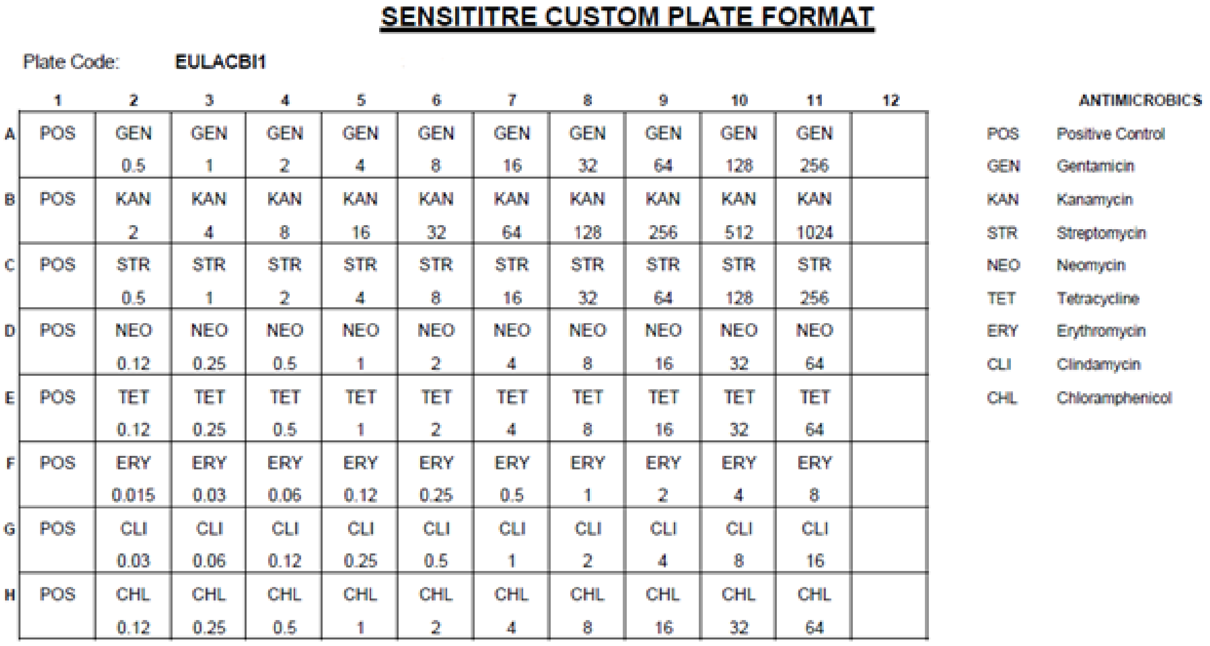
Figure 1: Diagram of the GPALL1F plate and antibiotic legend

	1	2	3	4	5	6	7	8	9	10	11	12
A	CHL 2	CHL 4	CHL 8	CHL 16	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	CLI 0.5	CLI 1	CLI 2
B	DAP 0.5	DAP 1	DAP 2	DAP 4	OXA+ 0.25	OXA+ 0.5	OXA+ 1	OXA+ 2	OXA+ 4	STR 1000	DT1	DT2
C	GEN 2	GEN 4	GEN 8	GEN 16	AMP 0.12	AMP 0.25	AMP 0.5	AMP 1	AMP 2	AMP 4	AMP 8	FOXS 6
D	LZD 1	LZD 2	LZD 4	LZD 8	PEN 0.06	PEN 0.12	PEN 0.25	PEN 0.5	PEN 1	PEN 2	PEN 4	PEN 8
E	RIF 0.5	RIF 1	RIF 2	RIF 4	VAN 0.25	VAN 0.5	VAN 1	VAN 2	VAN 4	VAN 8	VAN 16	VAN 32
F	SXT 0.5/9.5	SXT 1/19	SXT 2/38	SXT 4/76	LEVO 0.25	LEVO 0.5	LEVO 1	LEVO 2	LEVO 4	CIP 1	CIP 2	POS
G	SYN 0.5	SYN 1	SYN 2	SYN 4	TGC 0.03	TGC 0.06	TGC 0.12	TGC 0.25	TGC 0.5	NIT 32	NIT 64	POS
H	TET 2	TET 4	TET 8	TET 16	MXF 0.25	MXF 0.5	MXF 1	MXF 2	MXF 4	GEN 500	NEG	POS

Antimicrobics

CHL	Chloramphenicol
DAP	Daptomycin
GEN	Gentamicin
LZD	Linezolid
RIF	Rifampin
SXT	Trimethoprim / Sulfamethoxazole
SYN	Quinupristin / Dalfopristin
TET	Tetracycline
ERY	Erythromycin
OXA+	Oxacillin + 2% NaCl
AMP	Ampicillin
PEN	Penicillin
VAN	Vancomycin
LEVO	Levofloxacin
TGC	Tigecycline
MXF	Moxifloxacin
CLI	Clindamycin
STR	Streptomycin
CIP	Ciprofloxacin
NIT	Nitrofurantoin
DT1	D Test 1
DT2	D Test 2
FOXS	Cefoxitin screen
NEG	Negative control
POS	Positive control

Figure 2: Diagram of the EULACBI1 plate and antibiotic legend



SENSITITRE CUSTOM PLATE FORMAT

Plate Code: **EULACBI1**

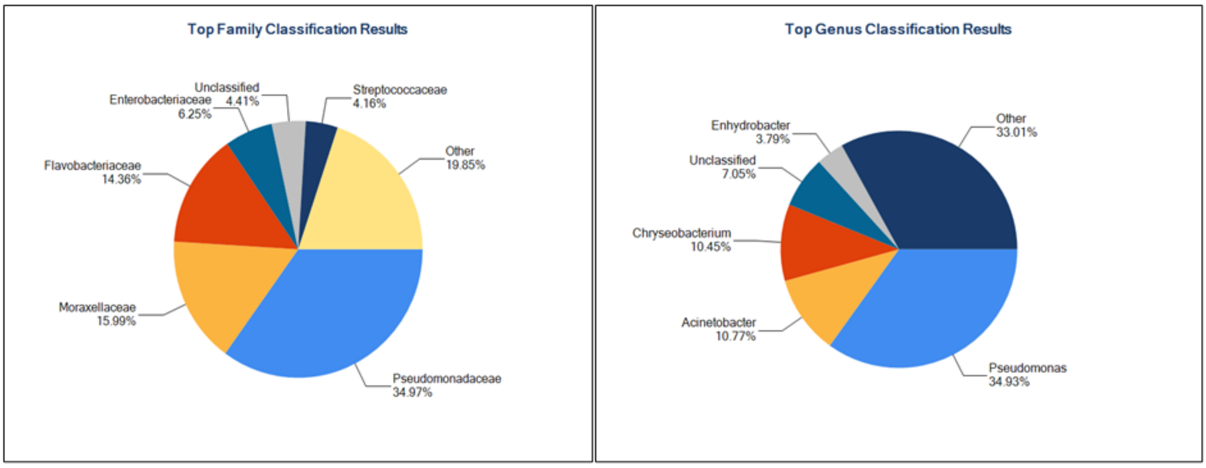
	1	2	3	4	5	6	7	8	9	10	11	12
A	POS	GEN 0.5	GEN 1	GEN 2	GEN 4	GEN 8	GEN 16	GEN 32	GEN 64	GEN 128	GEN 256	
B	POS	KAN 2	KAN 4	KAN 8	KAN 16	KAN 32	KAN 64	KAN 128	KAN 256	KAN 512	KAN 1024	
C	POS	STR 0.5	STR 1	STR 2	STR 4	STR 8	STR 16	STR 32	STR 64	STR 128	STR 256	
D	POS	NEO 0.12	NEO 0.25	NEO 0.5	NEO 1	NEO 2	NEO 4	NEO 8	NEO 16	NEO 32	NEO 64	
E	POS	TET 0.12	TET 0.25	TET 0.5	TET 1	TET 2	TET 4	TET 8	TET 16	TET 32	TET 64	
F	POS	ERY 0.015	ERY 0.03	ERY 0.06	ERY 0.12	ERY 0.25	ERY 0.5	ERY 1	ERY 2	ERY 4	ERY 8	
G	POS	CLI 0.03	CLI 0.06	CLI 0.12	CLI 0.25	CLI 0.5	CLI 1	CLI 2	CLI 4	CLI 8	CLI 16	
H	POS	CHL 0.12	CHL 0.25	CHL 0.5	CHL 1	CHL 2	CHL 4	CHL 8	CHL 16	CHL 32	CHL 64	

ANTIMICROBICS	
POS	Positive Control
GEN	Gentamicin
KAN	Kanamycin
STR	Streptomycin
NEO	Neomycin
TET	Tetracycline
ERY	Erythromycin
CLI	Clindamycin
CHL	Chloramphenicol

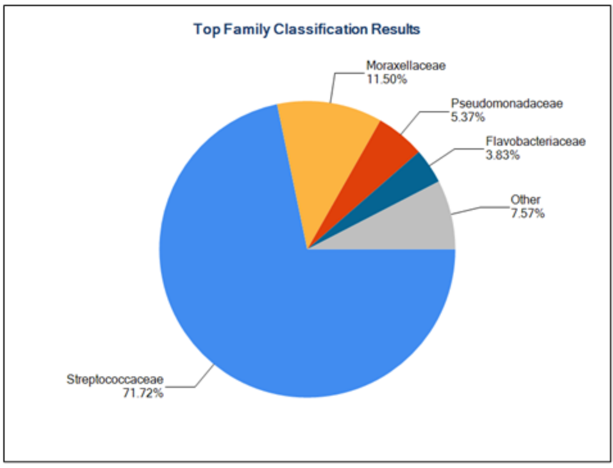
Figure 3: Results of the 16S rRNA metagenomic analysis of products from Dairy 2

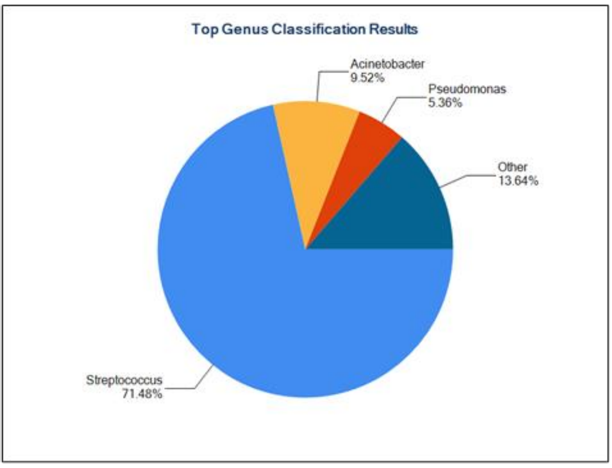
Summer sampling

Raw milk

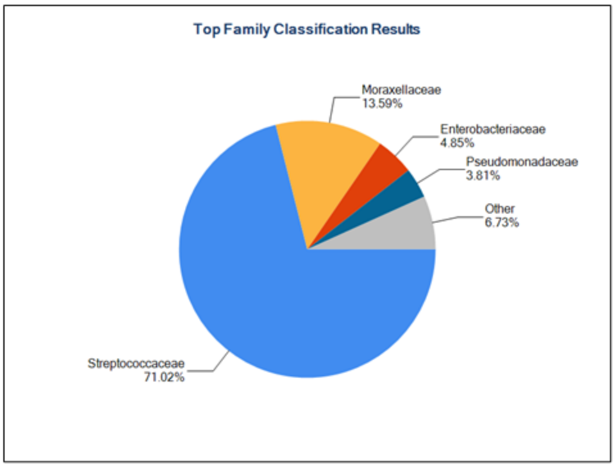


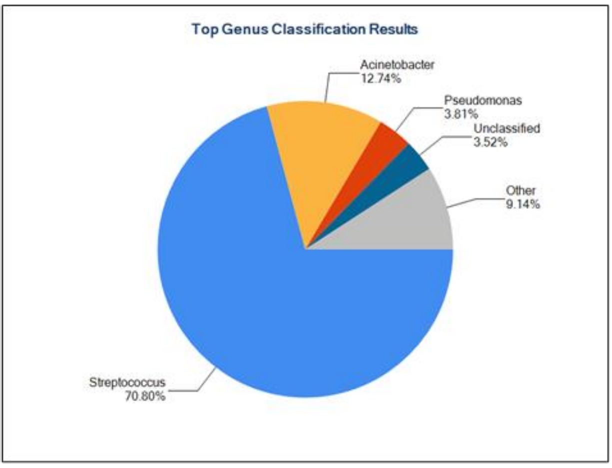
Curd



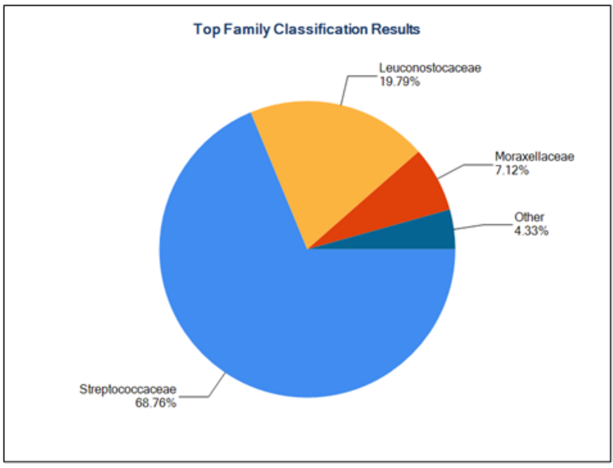


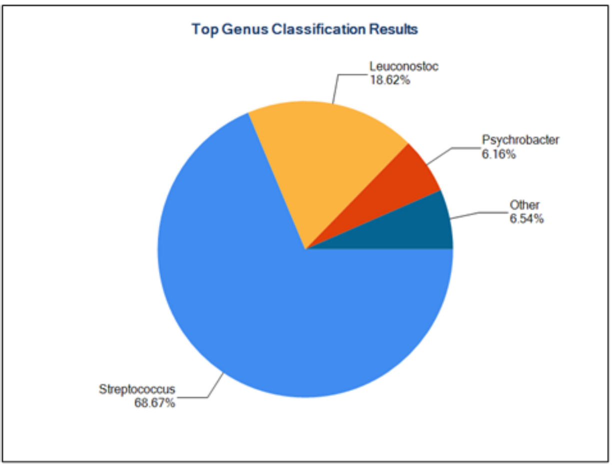
“Formaggio Maccagno” start of ripening





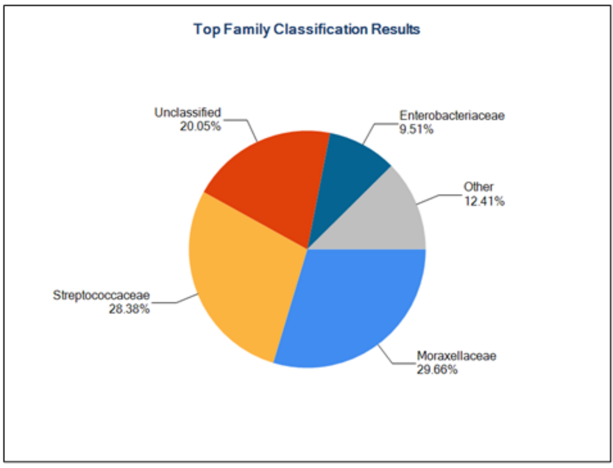
Final “Formaggio Maccagno”

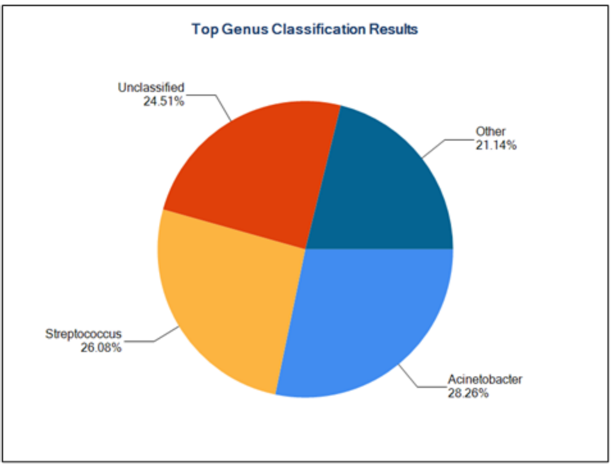




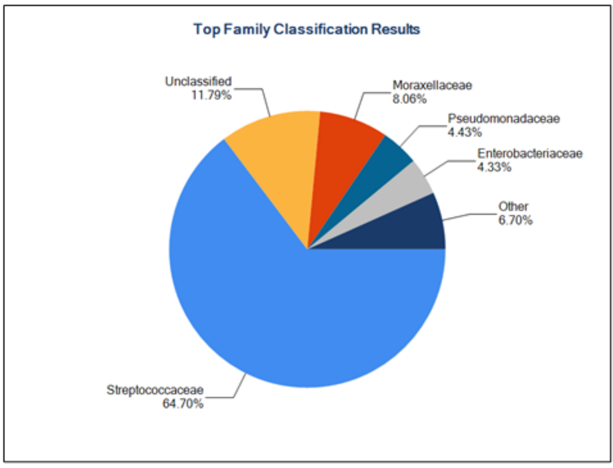
Autumn sampling

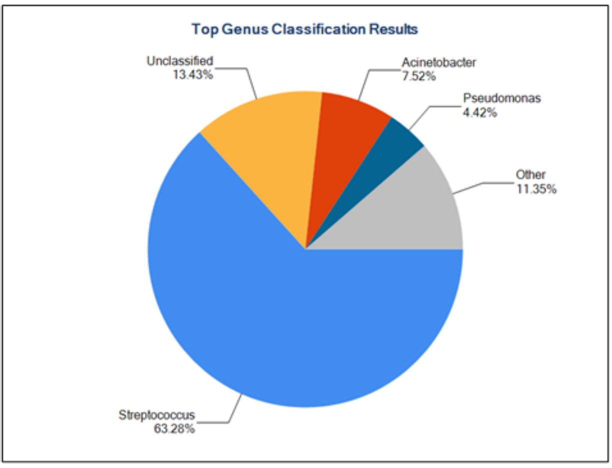
Raw milk



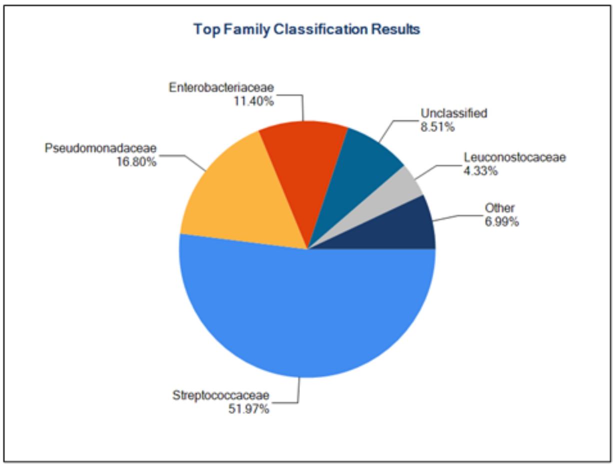


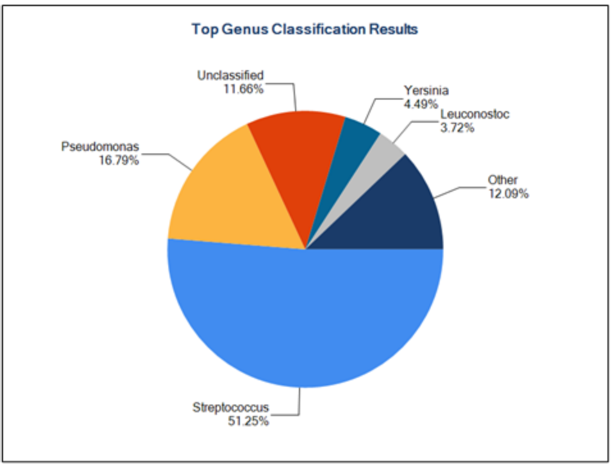
Curd





"Formaggio Maccagno" start of ripening





Final “Formaggio Maccagno”

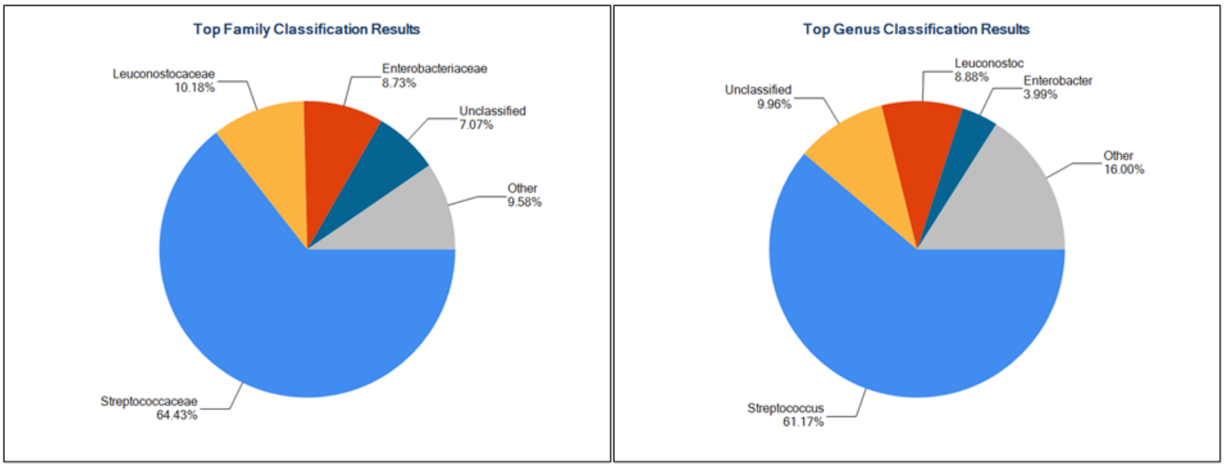
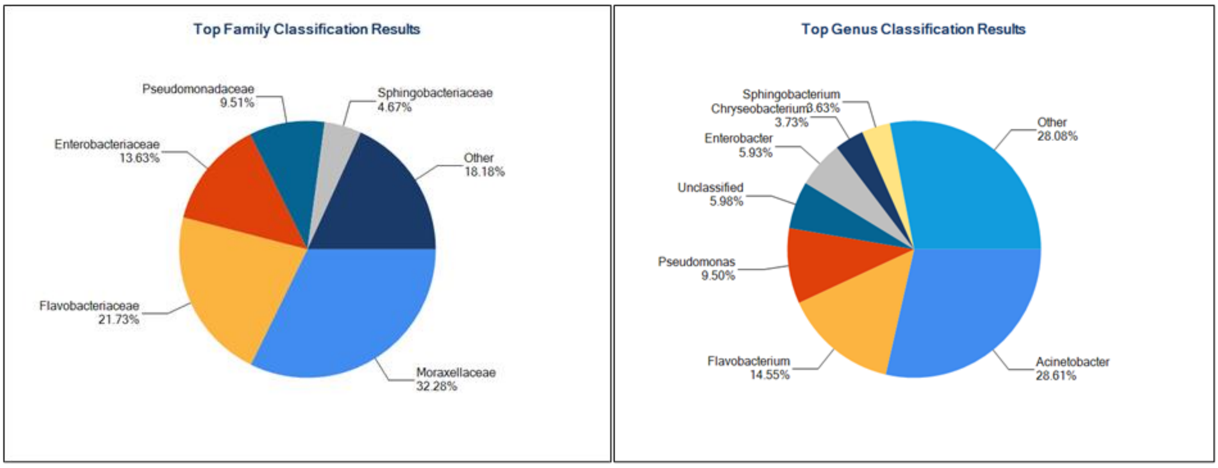


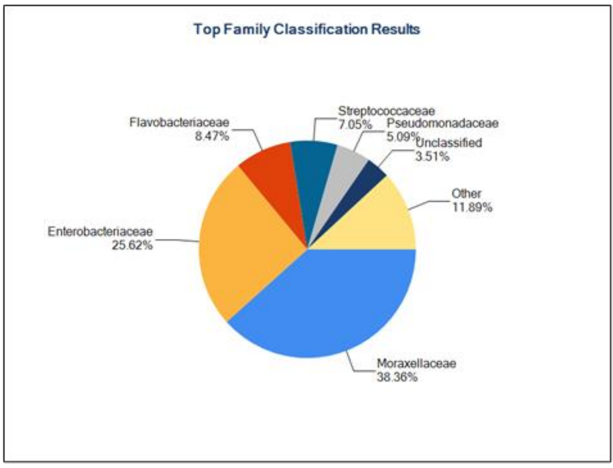
Figure 4: Results of the 16S rRNA metagenomic analysis of products from Dairy 3 in the two sampled seasons

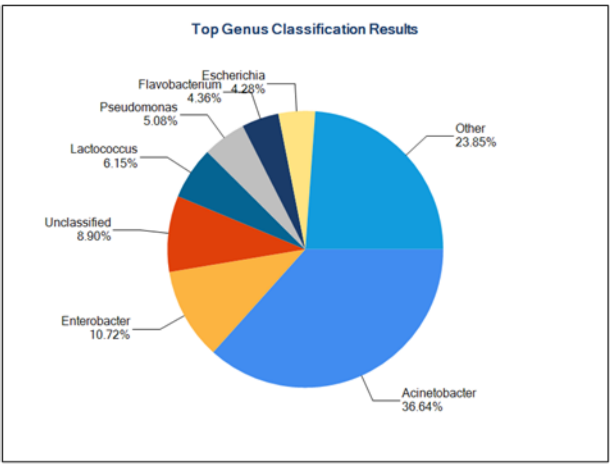
Summer sampling

Raw milk

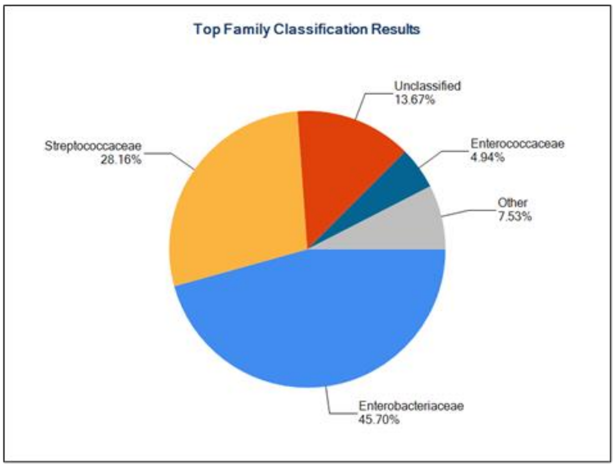


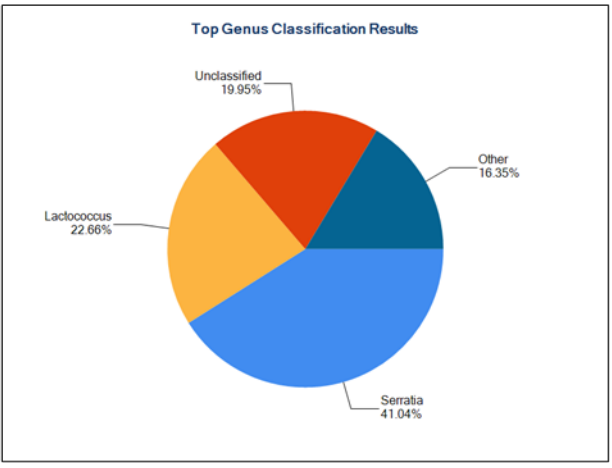
Curd





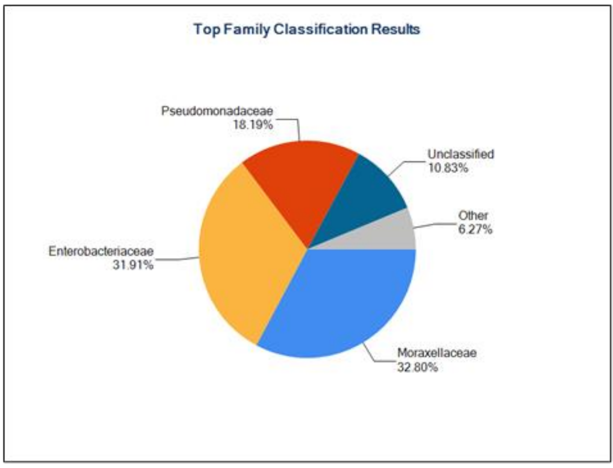
Cheese

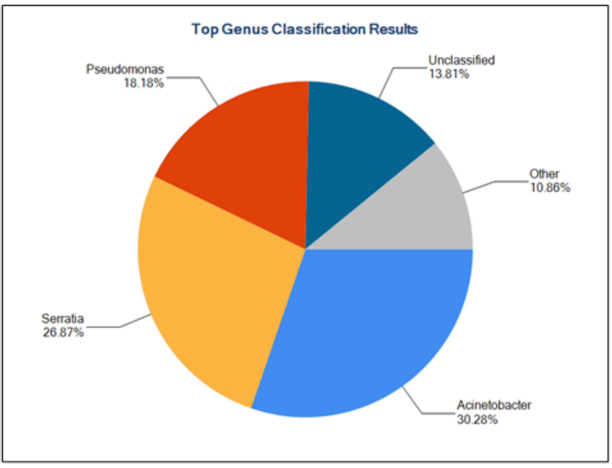




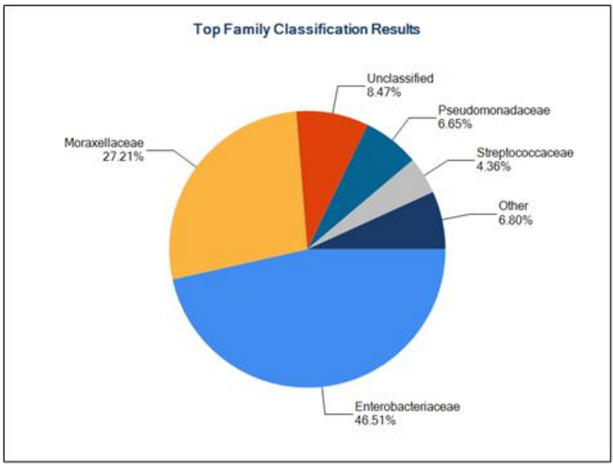
Autumn sampling

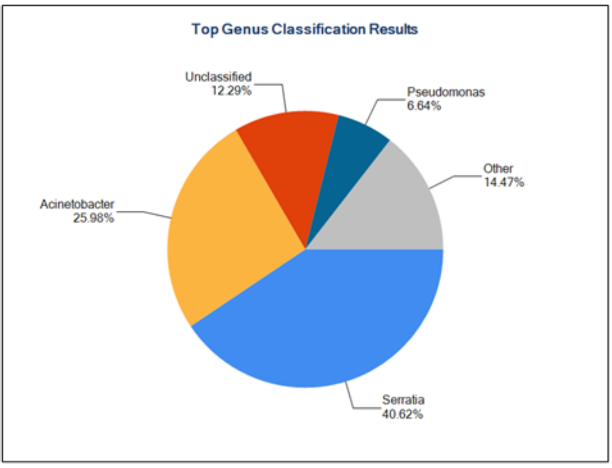
Raw milk



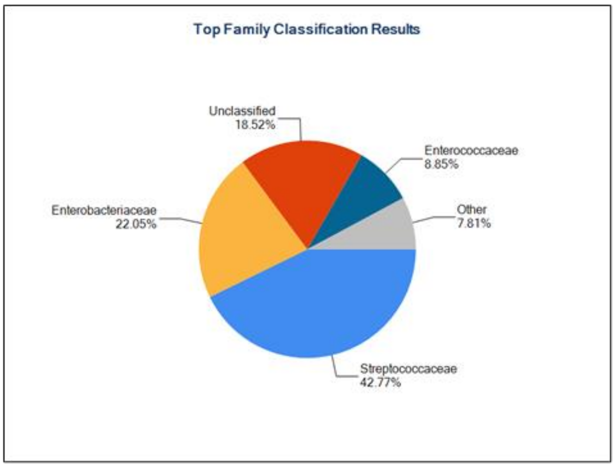


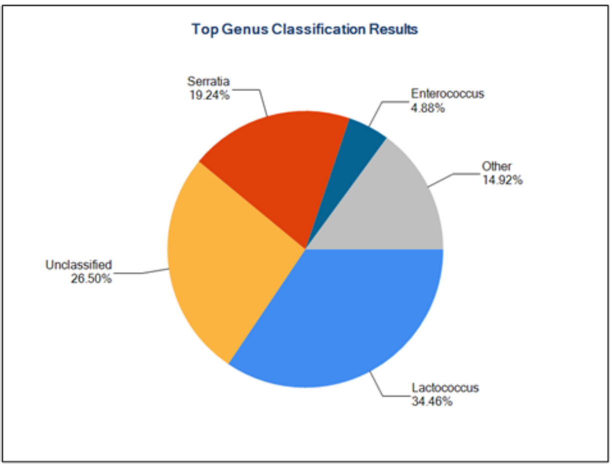
Curd


Top Family Classification Results
Unclassified 8.47%
Pseudomonadaceae 6.65%
Moraxellaceae 27.21%
Streptococcaceae 4.36%
Other 6.80%
Enterobacteriaceae 46.51%


Top Genus Classification Results
Unclassified 12.29%
Pseudomonas 6.64%
Other 14.47%
Acinetobacter 25.98%
Serratia 40.62%

Cheese


Top Family Classification Results
Unclassified 18.52%
Enterococcaceae 8.85%
Enterobacteriaceae 22.05%
Other 7.81%
Streptococcaceae 42.77%


Top Genus Classification Results
Serratia 19.24%
Enterococcus 4.88%
Other 14.92%
Unclassified 26.50%
Lactococcus 34.46%