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Understanding and detecting dairy ruminant mastitis: recent findings on microbial identification methods, diagnostic markers, and the microbiome

VET/05 – Infectious Diseases of Domestic Animals

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Al mio amatissimo nonno

Sommario

List of Abbreviations:

ACE - Abundance-Based Coverage Estimator

AGP - Alpha-1-Acid Glycoprotein

ALP - Alkaline Phosphatase

AMR - Antimicrobial Resistance

AMU - Antimicrobial Usage

APPs - Acute Phase Proteins

APR - Acute phase reaction

BDCT - Blanked Dry Cow Antibiotic Therapy

BDN - Banca Dati Nazionale

BHI - Brain Heart Infusion Broth

BM - Bulk Tank Milk

BSA - Bovine Serum Albumin

BTS - Bacterial Test Standard

BVDV - Bovine Viral Diarrhea Virus

BW - Body Weight

C.Re.N.M.O.C - Istituto Zooprofilattico Sperimentale della Sardegna/Centro di Referenza Nazionale Mastopatie Ovini e Caprini

CATH - Cathelicidin

CHCA - α-Cyano-4-Hydroxycinnamic Acid

CM - Clinical Mastitis

CMT - California Mastitis Test

CRP - C-Reactive Protein

DHB - 2,5- Dihydroxybenzoic Acid

DHI - Dairy Herd Improvement

EFSA - European Food Safety Authority

ELISA - Enzyme-Linked Immunosorbent Assay

ESBL - Extended Spectrum Beta Lactamase

ETs - Extracellular Traps (ETs)

FA - Ferulic Acid

FIL-IDF - International Dairy Federation

Hp - Haptoglobin

IBR - Infectious Bovine Rhinotracheitis

IG - Immunoglobulin G

IL6 - Interleukin 6

IL8 - Interleukin 8

IL10 - Interleukin 10

IL12 - Interleukin 12

IL1β - Interleukin 1β

IMI - Intramammary Infections

IQR - Interquartile Range

IZSSA - Istituto Zooprofilattico Sperimentale della Sardegna

LBP - Lipopolysaccharide-Binding Protein

LC-HRMS - Liquid Chromatography–High-Resolution Mass Spectrometry

LDH - Lactate Dehydrogenase

LF - Lactoferrin

LPS - Lipopolysaccharide

LS - Linear Score

m - Mass

m/z - Mass to Charge

MAA - Milk Amyloid A

MALDI-TOF MS - Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry

MBT - Maldi BioTyper

MRSA - Methicillin-Resistant *Staphylococcus aureus*

MS - Mass Spectrometry

NAG - N-Acetyl-β-D-Glucosamine

NAS - Non-*aureus* staphylococci

NASM - Non-*aureus* staphylococci and mammaliicocci

NCD - Neonatal Calf Diarrhea

NETs - Neutrophil Extracellular Traps

NM - Normal milk

NMC - National Mastitis Council

OTUs - Operational Taxonomic Units

PCR - Polymerase Chain Reaction

PCR–RFLP - Polymerase Chain Reaction-Restriction Fragment Length Polymorphism

PMNs - Polymorphonuclear Granulocytes

PRISMA - Preferred Reporting Items for Systematic Reviews and Meta-Analysis

QIIME - Quantitative Insights into Microbial Ecology

RE - Restriction Enzyme

RFLP - Restriction Fragment Length Polymorphism

RID - Radial Immunodiffusion

SA - Sinapinic Acid

SAA - Serum Amyloid A

SCC - Somatic Cell Count

SCFA - Short-Chain Fatty Acid

SCM - Subclinical Mastitis

SCs - Somatic Cells

SDCT - Selective Dry Cow Therapy

SPARCL - Spatial Proximity Analyte Reagent Capture Luminescence

STP - Serum Total Protein Concentration

SWiM - Synthesis Without Meta-Analysis

TGFα - Transforming Growth Factor α

TGFβ - Transforming Growth Factor β

TM - Transition Milk

TNFα - Tumor Necrosis Factor α

TOF - Time of Flight

WM - Waste Milk

WoS - Web of Science

z - Charge

Abstract:

Mastitis is one of the most economically impactful diseases in dairy farming. It may cause severe qualitative-quantitative losses in milk production, adversely affect animal welfare, and lead to early culling of animals. Mastitis can present itself in different forms, often due to the different causative agents. Two main manifestations of the disease are clinical, which is easier to detect due to the use of semi-quantitative techniques or simple examination of the udder, and subclinical , which has no obvious symptoms and requires milk testing to discover mastitis indicators. The focus of this thesis work is to understand dairy ruminant mastitis with a focus on recent findings on microbial identification methods, diagnostic markers of mastitis, and the impact of intramammary antibiotic treatment due to the presence of mastitis on the gut microbiome. The first study was focused on improving the identification of etiologic agents, focusing on the highly prevalent non-*aureus* staphylococci (NAS), with the application of PCR–RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) and MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) for improving the detection of intramammary infections (IMI). We applied MALDI-TOF MS and *gap* PCR–RFLP to 204 NAS and mammaliicocci (NASM) and to 57 streptococci isolated from the milk of sheep and goats with mastitis to compare their respective results. The study revealed a high level of agreement between MALDI-TOF MS and PCR–RFLP in identifying the most prevalent NASM and streptococci causing small ruminant mastitis. The second study aimed to conduct a systematic review to synthesize all the literature on the topic and improve the understanding of the diagnostic accuracy of protein marker-based immunoassays for mastitis detection. Currently, the most widely used tool for monitoring mastitis is the somatic cell count (SCC), expressed as the number of cells per ml of milk. Using SCC as a monitor has two major drawbacks. First, it has low specificity, and this limitation is particularly relevant in small ruminants, especially goats, as the SCC increases physiologically with parity and lactation number. The other major drawback is the difficulty of using this system to monitor mastitis in dairy herds of non-bovine species, for which clear reference values are unavailable. This has led to the search for new markers with higher specificity and suitability for easy measurements. Among the most studied are the protein molecules produced by the infected host in the context of inflammation and released in the milk, which makes them directly and easily detectable by techniques that exploit antigen-antibody interaction (immunoassay). To provide a general overview of their application in the different dairy species, we carried out a systematic review of the scientific literature using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines. Based on 13 keywords combined into 42 searches, we extracted 523 manuscripts from

three scientific databases. After screening for duplicates and pertinence, we summarized the main findings in 33 selected articles for the different markers. Where available, we reported their results in comparative tables, including sample selection criteria, marker values, and diagnostic performances. Finally, we reported the study limitations and bias assessment findings. The third study addressed the impact of mastitis on the calf gut microbiome, particularly concerning the impact of calf feeding with waste milk (WM). The risks associated with using WM as a feed source for calves have recently been highlighted. WM is a by-product of the dairy sector that, due to its physical, chemical, and microbiological properties and the presence of antibiotic residues from the treatment of lactating cows, cannot be marketed for human consumption. This milk may be a resource for calf feeding. Although WM represents an economically advantageous alternative and offers a viable solution to the environmental disposal of this product, it may present risks in terms of antibiotic resistance selection and may impair the healthy development of the calf gut microbiota. In particular, the administration of WM containing antimicrobial residues could contribute to select and maintain resistant bacteria in the animal's gut. In addition to presenting issues in terms of health and management of the animals themselves, this could pose a public health risk: calves, by becoming reservoirs of resistant bacteria, would contribute to the spread of these microorganisms through the environmental release of their feces. The trial assessed the longitudinal effect of unpasteurized WM containing residual cefalexin monohydrate on calf intestinal health and fecal microbiota in an 8-week trial. The feeding trial included 12 male Holstein Friesian calves. After colostrum, which lasted 3 days, 6 calves were fed waste milk (WM group), and 6 calves were fed normal bulk tank milk (NM group) for two weeks. At the end of this first phase, all calves were fed reconstituted whey powder and starter feed, according to standard farm protocols, for another six weeks. For the first two weeks, and then every two weeks until the end of the trial, calves were monitored and weighed, and fecal and swab samples were taken. Our results suggest that feeding pre-weaned calves with unpasteurized WM containing antibiotics leads to significant changes in the fecal microbiota composition, further discouraging this practice despite its short-term economic advantages.

Riassunto

La mastite è una delle patologie di maggior impatto economico nell'allevamento dei bovini da latte. Provoca gravi perdite quali-quantitative di latte, influisce negativamente sul benessere degli animali e spesso porta alla riforma precoce degli animali o, nei casi più estremi, all'abbattimento. Colpisce tutti i tipi di allevamento, da quello tradizionale a quello intensivo, ma i danni maggiori si riscontrano negli allevamenti con una gestione mirata a massimizzare la quantità e la qualità del latte, realtà in cui è necessario attuare piani di controllo per monitorare la situazione e consentire un intervento il più tempestivo possibile. La mastite può presentarsi in forme diverse, spesso dovute al diverso agente causale; si distinguono due manifestazioni principali della malattia: una forma clinica, più facile da individuare e per la quale è sufficiente l'utilizzo di tecniche semi-quantitative o la semplice valutazione visiva e tattile della mammella; e una forma subclinica, più insidiosa in quanto non presenta sintomi evidenti e rende necessaria la ricerca di indicatori direttamente nel latte. L'obiettivo di questo lavoro di tesi è la miglior comprensione della mastite nei ruminanti da latte, con particolare attenzione alle recenti scoperte sui metodi di identificazione microbica, sui marcatori diagnostici della mastite e sull'impatto del trattamento antibiotico intramammario dovuto alla presenza di mastite sul microbioma intestinale. Il primo studio si è focalizzato sul miglioramento dell'identificazione degli agenti eziologici, in particolare di quelli più problematici come gli stafilococchi non-*aureus* (NAS), con l'applicazione della PCR-RFLP (Polymerase Chain Reaction-Restriction Fragment Length Polymorphism) e della spettrometria di massa MALDI-TOF (Matrix-Assisted Laser Desorption/Ionization Time-Of Flight) per migliorare l'identificazione delle infezioni intramammarie (IMI). È stata applicata la spettrometria di massa MALDI-TOF e la PCR-RFLP *gap* a 204 stafilococchi non-*aureus* (NAS), e mammaliicocci (NASM) e a 57 streptococchi isolati dal latte di pecore e capre con mastite per confrontare i rispettivi risultati e identificare potenziali problemi e spiegazioni. I risultati ottenuti tramite le due tecniche, la spettrometria di massa MALDI-TOF e la PCR-RFLP, hanno rivelato un alto livello di accordo nell'identificazione dei NAS e degli streptococchi più diffusi che causano la mastite dei piccoli ruminanti. Il secondo studio aveva l'obiettivo di sintetizzare tutto il materiale presente in letteratura al fine di migliorare la comprensione dell'accuratezza diagnostica dei test immunologici basati su marcatori proteici per il rilevamento delle mastiti mediante una revisione sistematica. Attualmente, lo strumento più utilizzato per il monitoraggio della mastite è la conta delle cellule somatiche (SCC), un valore espresso come numero di cellule per ml di latte; tuttavia, presenta due principali deficit: non è un parametro con un'elevata specificità e questa limitazione è particolarmente rilevante nei piccoli ruminanti, soprattutto nelle capre, dove con l'avanzare della lattazione e del numero di parti, il numero di cellule somatiche (SCs)

rilevate nel latte aumenta fisiologicamente. Un altro grande svantaggio è la difficoltà di utilizzare questo sistema per monitorare la mastite nelle mandrie da latte di specie non bovine, per le quali non sono disponibili valori di riferimento chiari. Tutto ciò ha portato alla ricerca di nuovi marcatori con maggiore specificità e comunque caratterizzati da facilità di rilevazione: tra i più studiati attualmente ci sono le molecole proteiche prodotte dall'ospite infetto nel contesto dell'infiammazione e rilasciate nel latte, il che le rende direttamente e facilmente rilevabili con tecniche che sfruttano l'interazione antigene-anticorpo (immunoassay). Per fornire una panoramica generale della loro applicazione nelle diverse specie da latte, è stata svolta una revisione sistematica della letteratura scientifica utilizzando le linee guida PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis). Da tre banche dati scientifiche sono stati estratti 523 manoscritti, basati su 13 parole chiave combinate in 42 ricerche. Sono stati selezionati 33 articoli e i risultati sono stati riportati per i diversi marcatori sotto forma di tabelle comparative che includono i criteri di selezione dei campioni, i valori dei marcatori e le prestazioni diagnostiche, ove disponibili. Infine, sono stati riportati i limiti dello studio e i risultati della valutazione del bias. Il terzo studio ha affrontato l'impatto della mastite correlata ad altri aspetti come l'equilibrio del microbioma intestinale, in particolare ha valutato le conseguenze dell'utilizzo di latte di scarto (WM) ottenuto a seguito di trattamento intramammario con antibiotico per presenza di mastite. Il WM è un sottoprodotto del settore lattiero-caseario che, a causa delle sue proprietà fisiche, chimiche e microbiologiche, nonché della presenza di residui di antibiotici derivanti dal trattamento delle vacche in lattazione per diverse patologie, tra cui la mastite, non può essere commercializzato per il consumo umano. Questo latte può essere una risorsa per l'alimentazione dei vitelli, ma sebbene rappresenti un'alternativa economicamente valida e offra una soluzione praticabile allo smaltimento di questo prodotto nell'ambiente, può presentare rischi in termini di selezione della resistenza agli antibiotici. In particolare, la somministrazione di WM contenente residui antimicrobici potrebbe contribuire alla selezione e al mantenimento di batteri resistenti nell'intestino dell'animale. Questo, oltre a costituire un problema in termini di salute e gestione degli animali stessi, potrebbe rappresentare un rischio per la salute pubblica: i vitelli, diventando serbatoi di batteri resistenti, contribuirebbero alla diffusione di questi microrganismi attraverso il rilascio ambientale delle proprie feci. Lo studio condotto ha valutato l'effetto longitudinale del WM non pastorizzato contenente residui di cefalessina monoidrato sulla salute intestinale dei vitelli e sul microbiota fecale in una prova di otto settimane. Il trial ha compreso 12 vitelli maschi di razza Frisona Holstein. Dopo la colostratura, durata 3 giorni, 6 vitelli sono stati alimentati con latte di scarto (gruppo WM) e 6 vitelli con latte di vendita (gruppo NM) per due settimane. Al termine di questa prima fase, tutti i vitelli sono stati alimentati con siero di latte in polvere ricostituito e mangime pellettato (mangime starter), seguendo i protocolli standard dell'azienda, per altre sei settimane. Per le prime due settimane, e poi ogni due

settimane per il resto della sperimentazione, i vitelli sono stati monitorati e pesati, e sono stati prelevati campioni fecali e tamponi. I risultati suggeriscono che l'alimentazione dei vitelli in pre-svezzamento con WM non pastorizzato contenente antibiotici porta a cambiamenti significativi nella composizione del microbiota fecale, scoraggiando ulteriormente questa pratica nonostante i vantaggi economici a breve termine.

Introduction

Mastitis classification and definition

Mastitis due to intramammary infection (IMI) is a widespread disease in dairy cattle and accounts for 38% of dairy cattle's total direct costs of other common diseases (Radostits et al., 2000). Mastitis disease in dairy animals is significantly impactful, and its detection is, of high importance. Mastitis may be responsible for heavy economic losses due to reduced milk production (up to 70%), milk waste after treatment (9%), the cost of veterinary services (7%), and premature culling (14%) (Radostits et al., 2000). In addition to heavy milk quality and quantity losses, mastitis also causes irreversible damage to udder tissue and animal culling (Pol et al., 2007). Mastitis may cause a major animal welfare problem as it is associated with pain, reduced well-being and behavioral changes in affected animals (Medrano-Galarza et al., 2012).

Although mastitis is predominantly related to IMI of bacterial origin, during the 2012 National Mastitis Council (NMC) meeting, the definition of IMI provided in the International Dairy Federation (FIL-IDF) Bulletin No. 448/2011 clarified how the two terms are not synonymous, assigning a precise and different meaning to each (FIL-IDF, 2011). Specifically, IMI refers to infection of the mammary gland and its associated anatomical structures, i.e., the tissue secreting and containing the alveoli used for milk production. Mastitis is defined as an inflammatory condition that may affect one or more udder quarters, predominantly due to the invasion of pathogenic microorganisms (Lopez-Benavides et al., 2012). A schematic representation of the development of mastitis in an infected udder is represented in Figure 1.

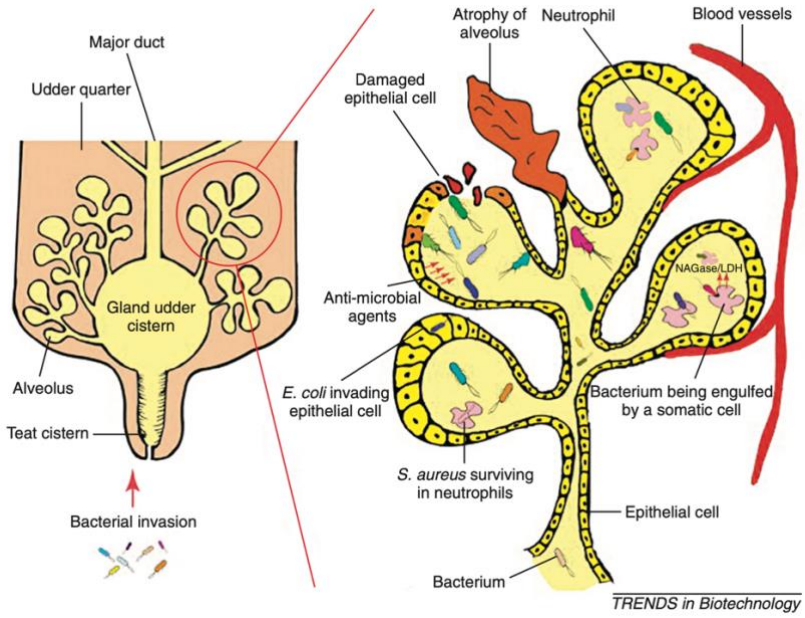

Figure 1: Development of mastitis in an infected udder (Reproduced from Viguier et al., 2009)

In most cases, mastitis is the consequence of IMI. That is, because of a bacterial infection of the mammary gland the host's immune system develops a response that initiates an inflammatory process, which in the specific case of the udder, is called mastitis (Figure 2).

	Intramammary Infection	Mastitis
International Dairy Federation definition	An infection occurring in the secretory tissue and/or the ducts and tubules of the mammary gland.	Inflammation of one or more quarters of the mammary gland, almost always caused by infecting microorganisms.
Diagnosis mainly by:	Bacteriological culture of milk samples obtained aseptically.	Subclinical: Measure of indicators of inflammation in milk samples such as somatic cell count (SCC) or California Mastitis Test (CMT). Clinical: Visual observation of milk and/or physical examination of the udder.

Figure 2: Definitions and characteristics of mastitis and IMI (Reproduced from Rollin et al., 2015)

The onset of infection is almost always consequent to the penetration of bacteria within the teat and mammary gland through the teat canal. Mastitis is a disease in which three factors interact: the host, the pathogen, and the environment (McDougall et al., 2009) (Figure 3).

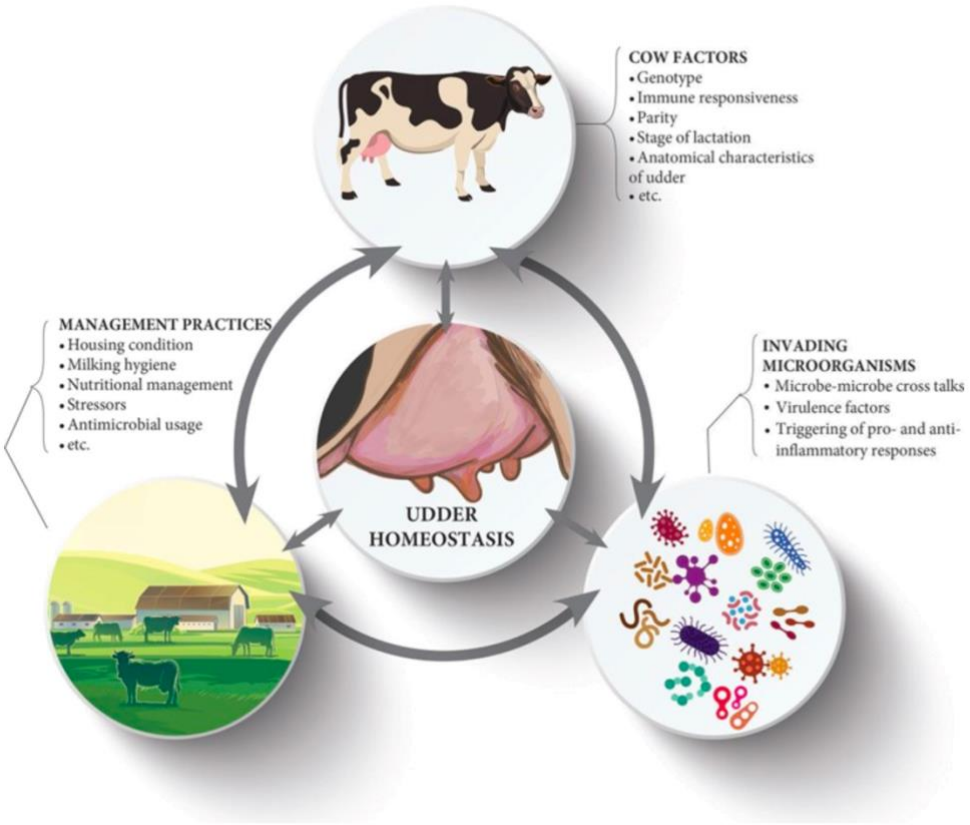

Figure 3: Factors that affect udder homeostasis and may promote the onset of IMI and, thus, mastitis (Reproduced from Derakhshani et al., 2018)

The host's immune response plays a crucial role in protecting the animal from developing an infection, inflammation, and the severity of the inflammatory process. Some phases of animal life, such as the days after calving, are known to be characterized by a decreased immune response. Management and genetic factors can also influence the immune competence of the animal. Environmental factors significantly influence bacterial survival and proliferation in the environment and the health status of their host animals (Schroeder, 2012). Management factors, including environmental hygiene or milking practices, can significantly influence the microbial load and the microbial populations on the farm, increasing animal exposure to pathogens. The tissue of the mammary gland is protected by two defense systems, innate or nonspecific immunity and acquired or specific immunity, that interact to provide protection from microorganisms that cause mastitis. The inflammatory response to infection allows mastitis to be classified into two types (Ruegg, 2011):

• Clinical mastitis (CM): characterized by a rapid evolution that causes the appearance of symptoms both at the level of the mammary gland and, depending on the microorganism involved, at the systemic level. The animal affected by this form of mastitis may present symptoms of a mild (abnormalities in color, consistency, and smell, with flakes or clots in milk) or severe (heat, swelling of the udder, abnormal secretions, fever, and loss of appetite) nature. Clinical mastitis is identified by a qualitative-quantitative worsening of milk, with which visible mammary gland changes are associated; the extent of these alterations allows us to distinguish three different types of clinical cases. Mild forms are characterized by macroscopically altered milk secretion, fibrin clots and frustules, watery consistency, and abnormal coloration. In cases of moderate severity, these manifestations are accompanied by mild swelling and soreness of the infected quarter. Severe forms are distinguished by a consistent reduction in milk production, up to complete agalactia, with general repercussions on the animal such as fever, loss of appetite, dehydration, and depression; this situation can lead to the death of the animal. In 10-15% of cases, mastitis arises suddenly and is characterized by a rapid course (Rasheed et al., 2020).

• Subclinical mastitis (SCM): causes few symptoms of general presentment and the udder and milk appear free of macroscopic alterations. SCM does not show any signs of local or systemic inflammation, as neither the udder nor its secretion is visibly altered. To detect this form of mastitis, it is necessary to examine milk composition and the production that may decrease with the increase of somatic cell count (SCC) (Cheng et al., 2020). This form is the most common; its prevalence is estimated to be 15 to 40 times higher than clinically visible mastitis.

Clinical and subclinical mastitis, if poorly treated, may evolve into chronic forms with clinical signs of persistent or recurrent character. Since the animal's immune system can limit but not eliminate the cause of the infection, the periodic appearance of an inflammatory response causes irreversible

damage to the mammary tissue, significantly increasing the risk of cow reformation (Benić et al., 2018). Another classification that distinguishes mastitis is based on the course of the disease: this, according to the severity and duration of infection, classifies mastitis as acute or chronic. Sudden onset characterizes acute mastitis, while chronic mastitis is characterized by an inflammatory process that can last several months (Rasheed et al., 2020) and can show clinical flare-ups at irregular intervals (Cheng et al., 2020).

Bovine udder health indicators

Mastitis is the main cause of increased cell content in milk. The cell types found in milk and generally defined as somatic cells (SCs) are mainly represented by epithelial cells and leukocytes (white blood cells). The latter include macrophages, lymphocytes, and neutrophils (polymorphonuclear granulocytes - PMNs). Neutrophils are the predominant cell type found in mammary tissues and secretions during inflammation, and in mastitis, they constitute more than 90% of the total milk leukocytes. Once at the site of infection, neutrophils phagocytize and kill the pathogens. Neutrophils exert their bactericidal effect through a respiratory burst that produces hydroxyl and oxygen radicals, which are important components of the oxygen-dependent killing mechanism. Another way neutrophils and other phagocytes and epithelial cells can disarm and kill extracellular pathogens is to release extracellular traps (ETs). Neutrophil extracellular traps (NETs) consist of a network of DNA, histones, antimicrobial proteins, and proteinases, which trap and inactivate invading microorganisms with no need for direct contact or engulfment by the host cell (Pisanu et al., 2015). Macrophages also play a central role in defending the udder. As well as in the induction of the immune response in case of bacterial invasion, they are responsible for the recruitment of neutrophils to the site of infection and a consequent cellular elevation in the milk secretion (Bronzo et al., 2020). If neutrophils move rapidly from the bloodstream and can eliminate the inflammatory stimuli (bacteria), then recruitment of neutrophils ceases, and the SCC returns to normal levels. If bacteria can survive this immediate host response, the inflammation continues, resulting in neutrophil migration between adjacent mammary secretory cells toward the alveolar lumen. The duration and severity of the inflammatory response has a major impact on the quantity and quality of milk produced. The effects of mastitis on milk yield are highly variable and depend on the severity of the inflammation, the causative agents and lesions produced, the efficacy of treatment, the production level, and the stage of lactation. Mastitis in early lactation causes a larger decrease in milk yield with long-term effects than mastitis in late lactation (Saunders, 2017). SCC is defined as the number of cells per milliliter of milk and is an essential parameter for detecting the presence of IMI; a value above 200,000 cells/ml in multiparous and 100,000 cells/ml in primiparous cows may indicate an initial inflammatory response.

In addition to the diagnosis of mastitis in the individual cow through milk analysis, cell counts measured in bulk milk can provide useful information on the health of the entire herd (Alhussien et al., 2018). The SCC of bulk tank milk has become a widely used test because it provides a valuable indicator of udder health and milk quality at the herd level. It is widely used to regulate whether milk may be legally sold and to determine the price paid for raw milk. Premium and penalty payments are calculated based on a 3-month geometric mean of weekly bulk milk tank SCC measurements. The bulk tank milk SCC is extremely useful in creating awareness of the existence of a mastitis problem so that when the SCC of bulk tank milk exceeds permissible limits, further herd investigation is indicated (Saunders, 2017). A level below 200,000 cells/ml is regarded as optimal (Ruegg et al., 2013). At the European level, the threshold of 400,000 cells/ml was imposed as the eligibility limit for human consumption by Regulation (EC) No. 853/2004. The SCC determined that bulk milk represents an average of the quality of milk produced on the farm, but even a few cows with high cell counts can result in high monthly variability. For this reason, Dairy Herd Improvement (DHI) programs resort to the linear transformation of SCC; the so-called Linear Score (LS) is based on the conversion of the SCC value to a logarithmic scale (Ruegg, 2003). SCC can be obtained by delivering the milk sample to the laboratory, where automated instruments analyze it. Indirect indicators include SCCs using automated electronic counters, the California Mastitis Test (CMT), increases in the electrical conductivity of milk, and increases in the activity of cell-associated enzymes (such as NAGase) in milk. Enzyme-Linked Immunosorbent Assay (ELISA) tests to detect neutrophil components have been developed but are not commercially available. Of these indirect tests, only the CMT and electrical conductivity can be assessed directly in the field, with CMT providing a more accurate screening test than electrical conductivity. The CMT visually assesses the thickening of milk caused by DNA released from lysed neutrophils, which is directly proportional to the concentration of leukocytes (Whyte et al, 2005). Alterations in mastitic milk also affect the pH, which increases from 6.6 to 6.9, and the conductivity of the milk. The change in electrolyte composition is due to higher concentrations of sodium and chlorine, which pass from the blood into the milk, and lower levels of lactose and potassium. In automatic milking systems, conductivity sensors have been introduced to measure the electrical conductivity of the samplefor early detection of subclinical cases. The significant spread of robotic milking has led to a gradual improvement in the detection of IMI and the development of biosensors to measure protein markers and non-protein molecules associated with mastitis. Inflammation markers are innovative diagnostic tools for the field, for which rapid agglutination, immunofluorescence, or lateral flow immunochromatographic assays have also been developed, and are useful for the laboratory also, where they are detected by ELISA or western immunoblotting. Among the most widely used enzymes for the diagnosis of mastitis are lactate

dehydrogenase (LDH), N-acetyl-beta-D-glucosamine test (NAGase), and alkaline phosphatase (ALP); among the nonenzymatic molecules, serum amyloid A (SAA), haptoglobin (Hp) and cathelicidin (CATH) represent the main proteins involved in the inflammatory process (Addis et al., 2016). It is important to understand that these indirect tests detect the presence of inflammation (subclinical mastitis) and not the presence of IMI.

Mastitis epidemiology

The microorganisms responsible for bovine mastitis, depending on their nature and the number in which they are present both in the environment and within the mammary gland, affect the immune response of the host and the course of the inflammatory state; the latter also depends on the state of health, age, parity, and the lactation stage of the animal. Although more than a hundred pathogens are known to be potentially involved in the infection process, staphylococci and streptococci are the most frequently isolated Gram-positive bacteria, and Enterobacteriaceae are the most common Gram-negative bacteria. They are generally classified into contagious and environmental species according to their primary method of transmission within the herd (Schukken et al., 2012).

Causative agents

Mastitis-causing bacteria can generically be classified as contagious (e.g., *Staphylococcus aureus, Streptococcus agalactiae, Mycoplasma* spp.) or environmental pathogens (e.g., *Escherichia coli*, *Enterococcus* spp., non-*aureus* staphylococci, *Streptococcus uberis*) (Cheng et al., 2020). Contagious pathogens are usually transmitted during milking by infected milk as the reservoir is the animal; these bacteria have high intrinsic pathogenicity, but in most cases, they can be eradicated from the farm. In the case of environmental pathogens, the reservoir is the environment. They are transmitted through contact with dirt, mud, and manure; these microorganisms do not possess high intrinsic pathogenicity but cannot be eradicated. Generally, some bacteria are more likely to induce clinical forms, such as *S. uberis, E. coli, Klebsiella* spp*., and Pseudomonas* spp., while other bacteria induce subclinical forms, such as *S. agalactiae* and non-*aureus* staphylococci (NAS); and other bacteria, such as *S. aureus*, can induce both forms (Benić et al., 2018).

Etiology and pathogenesis

The pathogens causing mastitis can be divided into contagious, whose main reservoir is an infected udder, and environmental, mainly from a contaminated environment (Benić et al., 2018). Mastitis is

a complex, multifactorial disease. For pathogens to penetrate the mammary glands, become established and cause infection, many factors such as hygiene, climate, milking management, nutrition, genetics, are involved (Rasheed et al., 2020). The environment and animals are rich in microorganisms, and milk is an excellent substrate for bacterial growth. To cause an infection, the causative agent must overcome the host's specific and nonspecific defense mechanisms. For this reason, it is usually easier for causative agents to enter through the teat canal. Only rarely does entry occur through the lymphatic or circulatory systems. The most significant risk for exposure occurs at the time of milking, during which the teat canal remains open for milk release and wider channels (sometimes selected for less milking time) may increase the risk of spontaneous milk loss and incidence of mastitis (Benić et al., 2018). During milking, the transfer of causative agents can occur between different animals or quarters of the same animal through the milking machine. Hygiene management at this stage and, in particular, the maintenance and cleaning of milking systems is critical. Since the teat canal is the first line of defense against bacterial invasion, teat damage or hyperkeratosis, i.e., the presence of a prominent smooth or coarse ring around the outer opening of the teat canal, are risk factors for the occurrence of infection (Benić et al., 2018). The entrance of bacteria into the mammary gland is due to their ability to overcome host defense mechanisms and adhere to and/or invade epithelial or immune cells (Rasheed et al., 2020). After penetrating and colonizing glandular tissue, bacteria multiply and produce metabolites. The affected tissues release inflammatory mediators that attract leukocytes, leading to an increase in SCC. Among other defense mechanisms, the host produces components with bactericidal activity (e.g., lysozyme, lactoferrin, peroxidase) as part of the innate immune response (Benić et al., 2018).

Main Infectious Pathogens

Contagious microorganisms include *S. agalactiae*, *S. aureus,* and *Mycoplasma* spp. The udders of infected animals represent the primary reservoirs of infection, which may be transmitted between infected and healthy animals or between quarters of the same animal through contaminated milk, especially during milking. At this stage, infectious agents from different sources, such as milkers' hands, materials used for teat cleaning, or milking system liners, enter the udder. The main procedures to control infections form spreadingwithin the herd include a proper milking routine teat disinfection at the beginning and end of milking and proper cleaning and regular maintenance of the milking system. The segregation of infected animals into differentiated groups which are milked last, their eventual reformation, and particular attention to preventing the introduction of infectious microorganisms into the herd through the entry of new animals are essential in farm management.

Since most infections with contagious microorganisms are contracted in the first two months of lactation and are responsible for subclinical forms that persist until the end of lactation, the control of contagious agents is of primary importance.

Streptococcus agalactiae is an important mastitis pathogen because of its highly contagious nature and ability to degrade milk quality. Most infected cows show no overt signs of disease, such as abnormal milk but have high SCC, decreased milk production, and a tendency to become chronic.

S. agalactiae is a highly contagious bacterium and obligate mammary pathogen capable of living exclusively in the udder, giving rise to predominantly SCM (Moroni et al., 2016). Once those infected cows have been identified and segregated for treatment or, if necessary, elimination, bulk milk samples from the farm should be analyzed monthly until the infection is completely eradicated. However, recent work has contradicted this premise by confirming that some strains of *S. agalactiae* may have a molecular epidemiology similar to that of an environmental pathogen (Cobo et al., 2018).

Staphylococcus aureus is responsible for causing the most common type of contagious mastitis in dairy cattle. Udders of infected cattle are the most common source for new infections but environmental sources do exist. These bacteria are also found on the skin of most cows and in the environment with another possible contamination source being flies. *S. aureus* is now considered the most important infectious pathogen, whose proliferation is strongly linked to colonization and invasion of the mammary gland. As with all contagious forms of mastitis, these bacteria are spread from cow to cow primarily during milking due to contamination of the milking system, milkers' hands, common towels, and other items. *S. aureus* commonly produces chronic infections that persist from one lactation to another despite dry cow therapy. The most common type of *S. aureus* infection is SCM and its subsequent evolution into a chronic form, which can persist from one lactation to the next and is characterized by the sporadic occurrence of clinical episodes; in the immediate postpartum, cases of hyperacute gangrenous mastitis may occasionally occur. The bacteria damage the udder tissue, the resulting involution of secretory tissue, formation of scar tissue, and development of abscesses, the rupture of which can cause infection in other areas of the mammary gland, can lead to irreversible damage (Peton et al., 2014).

***Mycoplasma* spp.** is a highly contagious but less common microorganism than *S. agalactiae* and *S. aureus.* Among the species recognized as responsible for IMI, *M. bovis* is the most pathogenic and most frequently isolated, followed by *M. alkalescens* and *M. arginini. M. bovis* is considered the most prevalent and clinically important mycoplasma species in dairy cattle that can also be readily transmitted from cow to cow during milking. Besides infected milk, another transmission route is respiratory since pulmonary forms and mastitis referable to *M. bovis* often coexist in the same herd (Gioia et al., 2021). A typical source of herd infection is purchased animals, especially non-lactating

heifers or cows subclinically infected with mycoplasmas. Infection of one or more animals causes the endemic spread of *M. bovis* within the herd. After calving, these animals may never develop CM but shed high levels of mycoplasma organisms in their milk or respiratory secretions. Although in most subclinical cases, the milk appears macroscopically normal with an inconstant cellular elevation, animals may develop acute forms with a tendency to become chronic; the early stages are characterized by massive destruction of the mammary secreting epithelium, which is followed by widespread fibrosis of the mammary parenchyma, and extensive alveolar atrophy. Outbreaks of mycoplasma mastitis are often seen several weeks after an outbreak of the respiratory disease in dairy herds. Without spontaneous bacteriological recovery, *M. bovis* mastitis is impossible to cure; segregation and culling of infected individuals is the only solution for its eradication.

Main Environmental Pathogens

The environmental microorganisms are represented by streptococci, including *S. uberis* and *S. dysgalactiae*, coliforms, and other Gram-negative bacteria, such as *E. coli, Klebsiella* spp., *Serratia* spp., *Enterobacter* spp., *Proteus* spp. and *Pseudomonas* spp. They form the microflora ordinarily present in cattle housing, especially in feces, soil, and bedding. As the number of bacteria in the housing environment increases or the animals' immune defenses decrease, the penetration of pathogens into the teat canal, especially in the first few hours after milking when the sphincter is still open, causes the onset of subacute clinical mastitis, usually of short persistence. Frequently these bacteria cause new infections during dry periods or at calving, when sudden but temporary acute clinical cases occur, often with spontaneous resolution. As a result of the emergence of certain strains with contagious characteristics, believed to be responsible for sometimes persistent infections, *S. uberis,* and *E. coli* have become increasingly important among the causative agents of mastitis. Strategies for controlling environmental pathogens focus on reducing the risk of infection by enforcing strict sanitation regulations (Krömker et al., 2014). Much of the focus for environmental mastitis control is on milking procedures in the milking parlor and keeping cows clean and dry in the barn, as infection can occur anytime between and during milking. Housing, bedding, and other surfaces in contact with cows should be clean and dry to limit the number of mastitis-causing pathogens in the cow’s environment.

Streptococcus uberis is one of the most frequently cultured streptococci from bovine udders. Animals become infected from environmental fonts between milkings when the teat ends in contact with surfaces contaminated with manure, soiled bedding, and mud. *S. uberis* is the most common cause of IMI contracted in the dry and pre-fresh periods. These bacteria may cause either CM with abnormal

milk, swelling of the gland, and fever or SCM with no apparent signs. *S. uberis* is a ubiquitous microorganism capable of colonizing animals and their environment. Infection is associated with bedding hygiene and milking routines when contagious transmission between healthy and infected individuals can occur. The clinical or subclinical outcome, the persistence of IMI, and the host immune response depend on the infecting strain (Schukken et al., 2011).

Streptococcus dysgalactiae exhibits both contagious and environmental pathogen characteristics. These bacteria are transmitted primarily during milking and can exist in infected mammary glands. Other sources of *S. dysgalactiae* are teat lesions and cattle tonsils, mouth, and vagina. *S. dysgalactiae* multiply at the teat orifice and readily colonize teat injuries. These organisms can be controlled during lactation by milking time hygiene, and usually, these are rapidly resolving forms, where only 20% become chronic (Schukken et al., 2011).

Escherichia coli is a Gram-negative bacterium (coliform) commonly found in bedding, manure, water, and soil that can cause life-threatening illnesses. Most of these infections happen during the first two weeks before calving through the first two months of lactation. Infections occur when the teat end contacts contaminated material between milkings. *E. coli* mastitis is commonly transient and associated with a rapid and intense inflammatory response in the cow, which can successfully clear the infection without causing permanent damage to the mammary tissue. In severe clinical cases, hypocalcemia may be seen, accompanied by anorexia, ruminal stasis, fever, debilitation, and dehydration; endotoxic shock may lead to the animal's death. Sometimes, recurrent or persistent cases can also be observed with alternating clinical and subclinical episodes associated with a significant drop in milk production. Since *E. coli* is present in cattle housing environments, the use of bedding consisting of inorganic material, preferably sand that is always clean and dry, appears to be the best solution to reduce the environmental bacterial load and, consequently, the exposure of teats to the pathogen (Schukken et al., 2011).

Minor Pathogens and Opportunists

Non-*aureus* staphylococci (NAS) are the major opportunistic type microorganisms in the bacterial microbiota of cattle, mainly located on the udder and teat skin, and are the most frequently isolated from bovine milk samples (Piepers et al., 2007; Sampimon et al., 2009; Mørk et al., 2012). They are a relevant cause of SCM in dairy herds that have successfully controlled major mastitis pathogens such as *S. aureus*, *S. uberis*, *S. agalactiae*, *S. dysgalactiae*, and coliforms (Schukken et al., 2009). *Staphylococcus chromogenes* is the most frequently isolated species from milk samples, followed by *S. simulans*, *S. haemolyticus*, *S. xylosus,* and *S. epidermidis* (Vanderhaeghen et al., 2014). Ecological,

epidemiological, and virulence behavior, as well as the antimicrobial resistance profile, vary greatly between species (Vanderhaeghen et al., 2014; Nobrega et al., 2018) and even between strains of a given species (Leroy et al., 2015; Piccart et al., 2016). Multiple studies demonstrate that NAS are the most prevalent bacteria found on teat apices of lactating dairy cows (De Visscher et al., 2014; Braem et al., 2013). They are also frequently isolated from teat canal samples (Taponen et al., 2008). These results suggest that colonization of the teat apex and canal may act as a reservoir for NAS species causing IMI. The importance of the teat canal as a barrier against bacterial invasion was supported in part by two recent studies showing that, collectively, NAS were more frequently found in samples obtained from quarter milking than those collected directly from the udder cistern or using the cannula technique (Hiitiö et al., 2016; Friman et al., 2017). Mastitis due to these pathogens occurs because of a drop in udder immune defenses, especially in primiparous cows at the beginning of lactation (De Vliegher et al., 2012). In addition to a lowered immune system, other factors that may contribute to the contamination of bulk milk are the presence of possible issues in the milking system, as well as inadequate udder preparation before or poor teat disinfection after milking operations.

***Prototheca* spp.** are algae isolated from plants, soil, mud, ponds and standing water, manure, and water troughs and can cause mastitis in dairy animals. These bacteria may cause acute or chronic mastitis, and clinical signs may be apparent, or the infection may remain subclinical. Affected animals may show decreased milk production and a watery mammary secretion with flakes and clots. Infections can come from teat end contact with contaminated surfaces during milking or be transferred from animal to animal at milking. *Prototheca* spp. is a unicellular alga to which several species belong, among which *P. bovis*, *P. wickerhamii,* and *P. blaschkeae* are those most commonly responsible for infections in animals (Ricchi et al., 2013). Recently, there has been an increase in mild to moderate clinical cases, while acute forms are more sporadic. Since mastitis from *Prototheca* spp. does not spontaneously heal in either lactation or dry period, and is refractory to antibiotic treatment, culling of the infected animal often remains the only solution.

Pathogen Identification

The microbiological analysis of milk allows for the isolation and identification of mastitis causative agents. The milk can be cultured as part of a herd examination for mastitis, as individual quarter or composite samples. Some farms prefer quarter samples at dry-off t to reduce costs as only affected quarters are treated (Saunders, 2017). Within a cow, the four quarters are not independent in relationship to IMI or subclinical mastitis; if one quarter is infected, chances are likely that one or more of the remaining quarters are infected or have SCM (Berry et al., 2006). Based on this, when

treating subclinical IMI at dry-off it makes more sense to treat the cow (i.e., all four quarters) and not just specific quarters. A definition of an IMI is ≥1 CFU/10 µL (Dohoo et al., 2011; Reyher et al. 2011). When collecting milk samples for culturing, strict attention paid to hygiene is important for avoiding sample contamination. The method of cleaning the teat is important. If the teats are dirty, they must be washed and well dried, to prevent water from entering the teat end and contaminating the milk sample. Teat ends are usually cleaned with gauze dipped in 70% alcohol followed by teat stripping. Two or three streams of milk are eliminated because cell and bacterial counts likely reflect the microbial situation within the teat rather than within the udder. With any delay between the collection of samples and laboratory analysis, the specimens should be refrigerated or frozen. Freezing milk samples seems to have variable effects on bacterial counts, depending on the bacteria. *Trueperella pyogenes* and *E. coli* counts are decreased by freezing, NAS counts are increased, and *Streptococcus* and *S. aureus* counts are either unaffected or improved. The laboratory analysis for pathogen isolation involves seeding milk samples onto culture media and can be time-consuming and expensive. Blood agar is the most suitable culture medium and allows easy and rapid microbial growth in 24-48 hours. The milk sample is considered contaminated when three or more species of bacteria are isolated. A significant advance in mastitis pathogen identification is provided by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (MS). MALDI-TOF MS on isolated bacterial colonies provides reliable genus and species information and is successfully applied to bacteria isolated from bovine milk (Nonnemann et al., 2019). Bacteriologically negative culture in cows with CM is a diagnostic problem. Even when milk samples are collected correctly, and bacteriologic culture is done using appropriate laboratory methods, 15% to 40% of samples from CM episodes result as bacteriologically negative. The negative results of these samples may be because of spontaneous elimination of infection, a low concentration of pathogens in the milk, intermittent shedding of the pathogen, intracellular location of the pathogens, or the presence of inhibitory substances in the milk. When no bacterial pathogen can be isolated from cases of CM using standard culturing techniques, ELISAs can be used to detect antigens against *S. aureus, E. coli, S. dysgalactiae,* and *S. agalactiae.* Traditional methods are complemented by molecular techniques based on nucleic acids. The Polymerase Chain Reaction (PCR) has brought important advantages to identifying bacterial species as it is possible to obtain accurate and reliable results in a shorter time. The test has potentially valuable advantages of greater sensitivity and faster time to produce a definitive result. The major disadvantages are cost (typically more expensive than routine milk culture), the inability to determine whether bacterial remnants detected by PCR in a milk sample reflect the presence of viable bacteria, and the lack of susceptibility testing results. Currently PCR tests provide their highest value in the routine testing of bulk tank milk for pathogen surveillance

and research studies related to CM episodes with no bacterial growth on culture (Keane et al., 2013; Hiitiö et al., 2015; Koskinen et al. 2010; Taponen et al. 2009; Cantekin et al., 2015).

Mastitis and economic impact

Although mastitis occurs sporadically in all species, its economic impact is higher in dairy cattle and may be one of the most expensive diseases in dairy herds. The losses caused by mastitis at the dairy industry level are estimated to be $1.7-2 billion annually in the United States, with one out of three cows affected by this disease (Misra et al., 2018). It is also estimated that the incidence of each CM in the US in the first 30 days of lactation costs about $444 to the farmer, while in Sweden, it shows a cost of about € 97/cow (Rasheed et al., 2020). Economic losses fall into several categories and can be classified as follows:

- Direct losses: costs for drugs, veterinary services, discarded milk, additional care for sick animals, and in some severe cases, culling or death of the animal (Benić et al., 2018).
- Indirect losses: decreased milk production, decreased milk quality due to changes in its chemical content, shorter productivity duration, and earlier dry-out (Benić et al., 2018). Mastitis is further associated with other problems, such as reproductive disorders and decreased feed intake, which may contribute to reduced milk production (Benić et al., 2018).

There are additional costs such as antimicrobial residues in milk from treated cows, milk quality control, dairy food manufacturing, the nutritional quality of milk, degrading of milk supplies caused by high bacterial counts or SCC, and interference with the genetic potential of some cows from early involuntary culling because of chronic mastitis (Petrovski et al., 2006). The total annual cost of mastitis in the dairy cattle population is estimated to be 10% of the total value of farm milk sales. The expenses of bovine mastitis can be divided into two main categories: production losses and control-related costs. Economic losses due to mastitis can be defined as a reduction in milk production due to this disease. The production and economic losses are usually divided into those associated with subclinical and clinical mastitis.

Subclinical mastitis: For many cattle farms, SCM is thought to be the most economically significant type of mastitis because of the long-term effect of chronic infections on total milk yield (Azooz et al., 2020). Around 75% of the economic loss from SCM is attributable to loss of milk production and a decrease in quality, compounded by the costs of treatment and early culling. Total milk losses from quarters affected by SCM were estimated between 10% and 26%. Lower SCCs are correlated with higher milk production, and the average milk production in herds is expected to decrease by 190 kg per unit increase in linear SCC. Most estimates indicate that, on average, an affected cow is estimated

to lose 15% of its production for lactation, and an affected quarter results in a 30% reduction in productivity. In addition to these losses, there is an added loss of about 1% of total solids by changes in composition (reduction of fat, casein, and lactose and increase of glycogen, whey protein, pH, and chlorides), which interferes with production processes, and other losses include costs of treatment and increased culling rates.

Clinical mastitis: It leads to a marked decrease in milk production, which are much higher in early than late lactation (Hagnestam et al., 2007). Milk production losses are also greater for cows with more than one lactation than for first-lactation cows (Hagnestam et al., 2007) and could differ with pathogen type. Usually, cases of clinical Gram-negative mastitis have more milk loss than Gram-positive cases and other cases (Schukken et al., 2009). CM also decreases the duration of lactation and increases the possibility of culling, and clinically affected quarters may not completely recover milk production in the following lactations. The costs of CM in dairy herds have been estimated in several countries. In a 5-herd study in New York state in 2008, each CM case cost $71 per cow-year, with a mean cost per clinical episode of $179. The latter estimation was based on $115 for milk yield loss, $14 for increased mortality, and $50 for treatment-associated costs (Bar et al., 2008). Other factors that affect the loss associated with mastitis include age (the loss is highest in older cows) and when the CM occurs in the first 150 days of lactation. CM needs to be detected and treated by the farm employees, which requires time and drugs and will result in further discarded milk due to drug withdrawal time. Sometimes, more complicated mastitis issues may require a veterinarian's intervention, another expense to consider (Aghamohammadi et al., 2018).

Correlation between mastitis and antibiotic resistance

The abuse of antibiotics has led to the resistance of bacteria to drugs, and multidrug resistance has become more prominent. In 2014, the British government called for a meeting on global antibiotic resistance and of the following impact assessment (O'Neill, 2014). Without actions leading to a reduction in resistance, the report's author states that in 2050 approximately 10 million people will die from bacterial infections caused by bacteria with antimicrobial resistance (AMR). Both the human and veterinary medicine sectors must assume responsibility and confine the development of resistance to preserve antibiotics as an effective therapeutic measure. Besides the main important goal to reduce antimicrobial usage (AMU), the application of antibiotics with critical importance for human medicine must be avoided whenever possible (Krömker et al., 2017). Although microorganisms with AMR currently do not represent a relevant issue in dairy farming, optimization of AMU is needed. It has been suggested that using antimicrobial drugs in food animals could affect human health by

increasing the risk of antimicrobial residues or influencing the generation or selection of drug-resistant food pathogens (Yan and Gilbert, 2004). In addition to the significant economic losses associated with the disease, mastitis has serious zoonotic potential and has been associated with the increasing development and rapid emergence of multidrug-resistant strains globally (Yan and Gilbert, 2004; Pol et al., 2007). Currently, antibiotics are the first-line treatment for bovine mastitis. Antibiotics are becoming ineffective with increasing antibiotic resistance and the emergence of resistant organisms and additionally there is the problem of antibiotic residues, a public health issue. The increase in drug-resistant strains has led to increased antibiotic use, which will cause environmental pollution and threaten human health (Han et al., 2022). Generally, antibiotics used in veterinary medicine are the same or very similar to those used in human medicine. Tetracyclines constitute the most widely used antimicrobial class in animals, followed by macrolides, penicillins, sulfonamides, aminoglycosides, fluoroquinolones, and cephalosporins (Schwarz et al., 2001). The dairy sector is characterized by lower use of antimicrobials than other livestock sectors, some critical antibiotic molecules such as third- and fourth-generation cephalosporins may be used. Blanket dry cow antibiotic therapy (BDCT), the administration of antibiotic treatments to cows during the dry period(typically 45 to 60 days), continues to be used by many farmers, even though selective dry cow therapy (SDCT) is mandatory in dairy farming to reduce antimicrobial usage. New legislation introduced by the European Union restricted and regulated the prophylactic and metaphylactic use of antibiotics in January 2022 (Huey et al., 2021). BCDT is based on applying the drug to animal's udder, along with a sealant to protect the udder from environmental contact. Resistance has been found in Europe for major mastitis pathogens such as *E. coli, S. uberis, and S. aureus* (Ricci et al., 2017). It is estimated that about 68% of antibiotics used on dairy cattle farms are used to treat mastitis. Specifically, 24% of antibiotic substances are administered for treating CM in lactating cows and 44% for therapy treatments during the dry period (Kuipers et al., 2016). Mastitis represents a serious problem for livestock farms, and the most rational therapeutic approach involves using antibiotics specific to the pathogens. The timeliness of therapeutic intervention becomes critical to improve the chances of therapeutic success; in animal husbandry, therapy is often initiated before the laboratory result. The indiscriminate use of antibiotics can favor the emergence of resistant pathogenic strains both in animals, with consequent difficulties for their remediation, and in humans due to the passage of these bacteria through the food of animal origin (Pesce et al., 2013). By law, diseased animals must be treated properly to ensure animal welfare. The disadvantages of AMU include partly low cure rates, residues in milk that could cause resistance, and the demand for alternative treatment (Gomes & Henriques, 2016). Strategies for reducing AMU in dairy production can target either the new infection control or the reduction in antibiotic therapy in sick animals. To reduce AMU in dairy cows,

it is crucial to avoid udder diseases. The use of antimicrobials in dairy cows usually occurs twice. Primarily, CM in lactating cows is mostly treated by intramammary administration of antibiotic ointments to the mammary gland cavity (local treatment). In severe cases of mastitis, antibiotics are administered systemically also. Secondly, implementing local antibiotic therapy on the day of dry-off has shown significant progress in reducing mastitis and has enabled many dairy farms to eliminate specific pathogens from the herd. To reduce AMU promptly and efficiently in dairy cows, it is, necessary to identify possible solutions for mastitis and dry-off management. A multifactorial disease process characterizes bovine mastitis; potential prophylactic measures include reducing new infections and pathogens transmission by optimizing management standards, separation, and culling decisions. Udder health management is a continuous process for improvement, so further enhancements may improve the reduction in AMU gradually and continuously. Increasing immunocompetence is an option for the reduction in mastitis, as the clinical outcome of mastitis depends in part on the immunological status of the individual cow. Researchers have been working on effective vaccines to prevent bovine mastitis, but developed vaccines, for example, against *S. aureus* or *E. coli* IMI, produce only limited protection (Gomes & Henriques, 2016). Further research in this area is promising.

Mastitis biomarkers

Mastitis due to IMI is a prevalent and costly disease in dairy cows. Although SCC is the most widely used mastitis detection system, it merely indicates the presence of a localized state of inflammation. Unless combined with a bacteriological test, its therapeutic use is limited. Numerous factors unrelated to mastitis can influence the levels of SCs in milk, reducing its specificity, such as breed, stage of lactation, and parity (Bytyqi et al., 2010). The other major limitation of SCs is their lower specificity in species other than cattle. It is possible to detect their values, but they are a less suitable parameter for determining the presence of inflammation. The clinical evaluation of udder and mammary secretions is generally combined with the milk SCC and frequently accompanied by its bacteriological culture to identify the causative microorganism. Several non-enzymatic milk proteins, including milk amyloid A (M-SAA), haptoglobin (HP), cathelicidin (CATH), and lactoferrin (LF), have been investigated as alternative biomarkers of mastitis for their relationship with mammary gland inflammation. Immunoassay methods have been developed for detection with varying degrees of success. Two important reasons are driving research to develop new diagnostic techniques based on easily exploitable biomarkers for the diagnosis of mastitis. The first concerns the increasing need for early diagnosis: it is essential to detect the presence of inflammation rapidly, especially when it comes to subclinical mastitis. The increase in sheep and goat farms is the other factor driving the

search for new biomarkers. Over the past 50 years, the demand for sheep and goat dairy products has more than doubled, and in line with this trend, increases of 26% and 53%, respectively, are expected by 2030 (Pulina et al., 2018). This demand has consequently led to the change of the farming system from small-medium farms to intensive systems, with all the implications that this entails, including the higher density of animals, milking machines, and increased stress. Mastitis is increasing, with even more drastic losses: the economic damage related to the drop in production is much more severe in these species because the amount of milk produced is less than in cattle, andadditionally, the technological damage is also more significant. In the presence of mastitis, lactose value decreases and milk pH increases, hindering calcium activity during the clot formation process (De Olives et al., 2020). Considering that almost all sheep and goat milk is processed, the economic losses in the presence of mastitis are significant. To address this issue, the use of molecular biomarkers produced by the host during the immune response and detectable in milk is becoming more widespread in recent decades. These are mainly proteins synthesized by the mammary gland or other organs (such as the liver) and then delivered to the mammary gland through the blood system due to the increased permeability that the blood-mammary membrane undergoes due to a state of inflammation. These proteins do not have enzymatic activities and must be measured with immunoassay. In this way, easy-to-prepare tests can be taken advantage of to develop rapid tests that can be carried out directly on the farm, such as lateral flow tests (immunochromatographic systems), without the need to use expensive reagents or even ELISA tests to be carried out in the laboratory on frozen milk samples.
The key features that make these new diagnostic techniques viable alternatives to classical SCC are:
- Specificity: the molecules detected are produced exclusively when mastitis is present and are not influenced by other animal-related factors, as is the case with SCC;
- Release kinetics in milk: the proteins investigated are released immediately at the onset of the animal's immune response and then disappear at the end of it. They are not present in "healthy" milk, thus avoiding undesirable false-positive phenomena;
- Resistance to sample freezing: tests can be performed even on samples stored for several days without altering the components of interest;
- Easily repeatable tests (Addis et al., 2017).

Acute phase proteins and the innate immune response

Acute phase reaction (APR) is the physiological response to infections and injuries that involves local inflammation and the initiation of events leading to a systemic response. This multiplicity of changes is distant from the injury site. It includes fever, leukocytosis, and the quantitative and qualitative modification of a group of non-structural proteins present in the blood and other biological fluids, collectively called acute phase proteins (APPs) (Ceciliani et al., 2012). During the inflammatory processes of mastitis, the mammary gland is of particular interest since local APP synthesis is known to be induced, and APP can also be present in milk where they might also exert effects on the offspring (Ceciliani et al., 2012). Biomarkers of interest for mastitis detection are produced directly by the diseased subject following stimulation of the immune system. Following tissue damage or entry of a pathogen, the body emits chemical signals that activate the immune system; first, signs of inflammation *rubor* (redness), *tumor* (swelling), *calor* (increased temperature), and *dolor* (painful sensation) develop locally, together with leukocytosis and cytokine production. This so-called acute phase prevents the spread of the pathogen to other sites and simultaneously limits tissue damage, promoting the repair process to return the body to a state of homeostasis. Following the initiation of the acute phase response, cascading processes involving mediators of inflammation are activated, which in turn cause localized vascular and systemic effects involving several organs, including the liver. The latter, within hours, changes its biosynthetic pathways, beginning to produce acute-phase proteins (Simon et al., 2004), but a part of APPs are synthesized by epithelial and endothelial cells (Morrow et al., 1981) directly at the site of inflammation. To diagnose the presence of localized inflammation, it is sufficient to detect the concentration of the protein of interest both at the single organ level and in the circulation if the former is much higher than the latter, it will be a clear sign of localized inflammation. In our specific case, a microorganism that manages to overcome the first defense barriers, such as teat skin, and milk flow (Rosa et al., 2014), ascends inside the mammary gland where it finds the right conditions for its development and proliferation, thus establishing a true infection resulting in mastitis. Depending on the microorganism, the infectious load, and the animal's health status, there will be different types of mastitis (clinical, subclinical, and acute). No matter the type, the first phase of the acute response will be characterized by increased APP. Not all protein categories increase with the same intensity during inflammatory phenomena (Cray et al., 2009) (Figure 4). The definition of "positive APPs" is based on this principle. The term major denotes APPs that increase their blood concentrations by 10-1000 compared to physiological values, moderate those that increase 4-10-fold, and minor those APPs that increase by 2-3-fold (O'Reilly et al., 2014). This classification varies depending on the species under consideration.

Species	Major (>10-fold increase)	Moderate (1- to 10-fold increase)
Cat	α1-acid glycoprotein, serum amyloid A	haptoglobin
Chicken	none	α1-acid glycoprotein, ceruloplasmin, serum amyloid A, transferrin
Cow	haptoglobin, serum amyloid A	α1-acid glycoprotein, C-reactive protein, fibrinogen
Dog	C-reactive protein, serum amyloid A	α1-acid glycoprotein, ceruloplasmin, haptoglobin
Goat	haptoglobin, serum amyloid A	fibrinogen
Horse	serum amyloid A	fibrinogen, haptoglobin
Human	C-reactive protein, serum amyloid A	α1-acid glycoprotein, fibrinogen, haptoglobin
Mouse	haptoglobin, serum amyloid A, serum amyloid P	C-reactive protein, fibrinogen
Nonhuman Primates	C-reactive protein	α2-macroglobulin, fibrinogen, serum amyloid A
Pig	haptoglobin, serum amyloid A, major acute phase protein	α1-acid glycoprotein
Rabbit	haptoglobin, serum amyloid A	α1-acid glycoprotein, C-reactive protein, fibrinogen
Rat	α1-acid glycoprotein, α2-macroglobulin	C-reactive protein, fibrinogen, haptoglobin
Sheep	haptoglobin, serum amyloid A	α1-acid glycoprotein, C-reactive protein

Figure 4: Species-specificity of major and moderate acute phase proteins (Reproduced from Cray et al., 2009)

Although the common purpose of all APPs is to restore the body to a state of homeostasis as quickly as possible, each possesses a different mechanism of action. Here is the description of the four most frequently mentioned protein markers in scientific literature on mastitis.

Milk Amyloid A

Amyloid A (MAA) is an acute-phase protein synthesized by mammary gland epithelial cells; it is an isoform of Serum amyloid A (SAA) synthesized by hepatocytes during the acute-phase response: the former is detected directly in milk, while the latter is found primarily in blood and can be detected in milk because of increased permeability of the blood-mammary barrier but is less specific.
It is a major APP, as its levels increase 1000-fold following inflammation and then quickly return to barely detectable values as soon as the cause is resolved (Taghdiri et al., 2018). MAA is synthesized by epithelial cells, stimulated by mediators of inflammation such as Interleukins (Brenaut et al., 2014) or directly by bacterial components, for example, by lipoteichoic acid, a component of the wall of Gram-positive (Weber A. et al., 2006) or the lipopolysaccharide (LPS) present in the membrane of Gram-negative bacteria (Yu G.M. et al., 2017). Although SAA in cattle had already been mentioned in studies from the 1970s (Gronlund et al., 2003), it was not until the early 2000s that the MAA isoform was identified and subsequently considered as a biomarker for the detection of mastitis, and MAA values depend only on what is happening inside the mammary gland (Taghdiri et al., 2018).

Haptoglobin

Haptoglobin (Hp) is also an acute-phase protein that can be detected in milk. Part of the Hp present within the mammary gland is synthesized directly by leukocytes and epithelial cells. In contrast, the remaining portion originates in the liver and reaches the sites of interest via the bloodstream (Figure 5). As for the hepatic fraction, the synthesis mechanism follows that of SAA. A localized inflammatory process (in this case within the mammary gland) causes the production of pro-inflammatory cytokines that, at the hepatic level, induce an alteration of biosynthetic pathways resulting in the production of Hp and release into the bloodstream, from which, once it reaches and crosses the blood-mammary barrier, it passes directly to the milk in which its presence can be detected. Since it is a protein that is synthesized because of inflammatory stimulation of various kinds, it is not considered a specific protein in the absolute termIt can be regarded as specific for the diagnosis of mastitis. Its increase detected directly in milk is a sign of localized inflammation, and, as mentioned earlier, part of Hp is synthesized directly within the mammary gland. As with MAA, Hp synthesis is stimulated by pro-inflammatory molecules and bacterial components, such as lipopolysaccharide (LPS) (Schmitz et al., 2004).

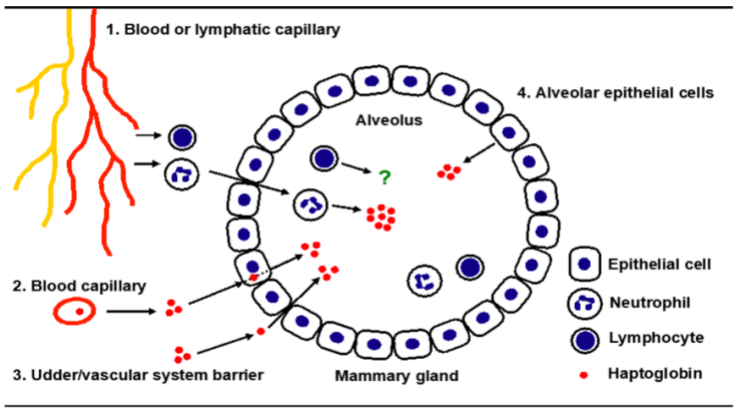

Figure 5: Different origins of haptoglobin. Some of it is synthesized in the liver and carried into the mammary gland through the bloodstream (2), some are synthesized directly by epithelial cells (4), and finally, a portion is produced directly by leukocytes in milk (5) (Reproduced from Lai et al., 2009)

Cathelicidin

Cathelicidin (CATH) is another protein widely studied as a marker of mastitis. Not belonging to the APP family, it is a protein involved in the innate immune response phase. Specifically, it is a family of cationic peptides that exert antimicrobial activity not only on Gram-positive and Gram-negative but also on many types of viruses and fungi (Reczyńska et al., 2018). The primary antimicrobial function of CATH is due to its ability to interfere with the pathogen's cell membrane structure (Agier

et al., 2015) through several mechanisms, including a *carpet model* involving the adhesion of multiple peptides at a specific point on the pathogen's membrane, causing structural disruption; *detergent model* in which peptides act as a solvent towards the membrane; *barrel-stave model* through which CATH causes the formation of pores on the bacterial/viral surface (Figure 6).

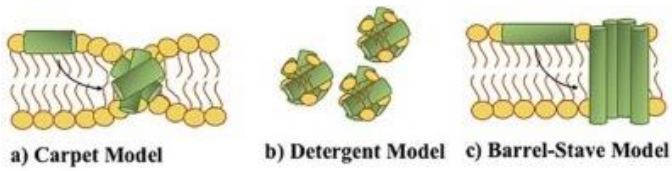

Figure 6: Main mechanisms of broad-spectrum action of cathelicidin (Reproduced from Young-Speirs M. et al., 2018)

Eight cathelicidins have been identified in cattle (Young-Speirs et al., 2018), five of which (Cath 1, Cath 2, Cath 3, Cath 6, and Cath 7) have also been identified in the goat species (Zhang et al., 2014). In 2011, one study reported a difference in CATH levels depending on the causative agent, hypothesizing that different microorganisms stimulated the synthesis of this peptide differently (Smolenski et al., 2011). Another study confirmed this theory, pointing out that different microorganisms cause different elevations of CATH levels but do not impair mastitis detection activity.We will have bacteria capable of stimulating greater CATH synthesis, such as *S. agalactiae*, and those that cause less elevation of values, such as NAS (Addis et al., 2017). When first encountering the pathogen, CATH can be produced by mammary alveolar epithelial cells. This phenomenon makes it an early and specific marker of mastitis (Cubeddu et al., 2017).

Lactoferrin

Although lactoferrin (LF) is not an acute-phase protein, some authors consider it to be such, given its characteristic of increasing exponentially during an inflammatory state (Kanyshkova et al., 2001). LF in milk was found to be one such reliable biomarker for the diagnosis of SCM (Sadek et al., 2016). It is a glycoprotein belonging to the transferrin family and is present in several body fluids, including milk. It is synthesized by different cells, mainly by neutrophils, in which it is stored within granules called "secondary" (Adlerova et al., 2008) and, in the case of our interest, by cells of the mammary epithelium (Masson et al., 1969). LF has been associated with several biological functions, including inhibition of several microorganisms, transport of iron, immune modulation, enhancing cell differentiation, and regulation of several other cell functions (Brock et al., 2002).

Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry (MALDI-TOF MS)

In recent years, MALDI-TOF MS (Matrix-Assisted Laser Desorption/Ionization Time-Of-Flight Mass Spectrometry) technique has become a reference method for the routine identification of bacterial isolates in clinical microbiology laboratories around the world. Its high specificity, ease of use, and cost-efficiency, together with its ability to provide reliable results in less than 5 minutes, have favored its implementation and further development (Oviaño et al., 2021). This technique can analyze different samples, such as solutions of organic molecules, nucleic acids, proteins, and whole microorganisms. The latter two applications are, at present, the most widely used in microbiology (Brunelli, 2016); contemporary scientific publications propose its use for the identification of pathogens directly from the clinical specimen and rapid screening of some mechanisms of antibiotic resistance (methicillin resistance; vancomycin resistance; extended-spectrum β-lactamase production). MALDI-TOF MS is a phenotypic technique that relies on the protein "fingerprint" of the bacterium obtained by mass spectrometry and is currently able to accurately identify, at the species level, most Gram-positive and Gram-negative bacteria; this technique is also used and quite accurate for the identification of yeasts, while for fungi (filamentous and dermatophytes) specific standardized procedures are required. One of the main advantages of its use is the reduction of the time required for identification from 24-48 hours to less than an hour (Croxatto et al., 2011). MS is an analytical technique that is used both for the identification of molecules and for quantitative determinations of known compounds. The principle on which it is based is the ability to separate a mixture of ions according to their mass-to-charge (m/z) ratio (Brunelli, 2016). This technique has been used for decades in chemistry. It was not until 1975 that two researchers, Anhalt and Fenselau, proposed using it for bacterial identification due to the uniqueness of the mass spectra of each bacterium allowing identification at the genus and species level and having potential for strain identification as well. MALDI-TOF MS was developed in 1987 by Hillenkamp, Karas, and Tanaka (Cembrola, 2005). The development and commercialization of spectrometers with robust and easy-to-use platforms have increased the uptake of this technology in many microbiology laboratories (Brunelli, 2016).

A mass spectrometer consists of three functional units:

- An ionization chamber with a laser source (Brunelli, 2016) for ionization and transfer of sample molecules into the gas phase (Croxatto et al., 2011);

- A time-of-flight (TOF) analyzer that separates ions based on the m/z ratio (MALDI generates mainly single-charge ions;, it can generate spectra with more proteins than those derived from other techniques);
- A detector of the separated ions (Croxatto et al., 2011).

MALDI-TOF MS can be used to analyze intact proteins with the formation of small fragments, and it is this property that makes it suitable for detecting protein mass spectra of whole organisms (Brunelli, 2016). In the analysis, the sample is mixed with a matrix that determines its crystallization; the latter is composed of small acid molecules with strong optical absorption in the wavelength range of the laser used (e.g., 2,5-dihydroxybenzoic acid (DHB), α-cyano-4-hydroxycinnamic acid (CHCA), sinapinic acid (SA), ferulic acid (FA), 2,4-hydroxy-phenylbenzoic acid) (Croxatto et al., 2011). The choice of the matrix is also influenced by its ease of sublimation (direct transition from solid to vapor state) and intense absorbance at the laser wavelength (Brunelli, 2016).

Generally, microorganisms can be analyzed by MALDI-TOF without pretreatment, and only in the case of some more resistant ones are strong organic acids and/or alcohols added in the pretreatment steps; in other cases (e.g., Actinomyces) specific pretreatment or protein extraction procedures can be used (Bizzini et al., 2011). In all cases, following the correct procedures and manufacturer's recommendations is essential to achieve accurate identification (Croxatto et al., 2011). One of the most widely used practical approaches is to take fresh colonies of bacterial isolates from the agar medium (after 18-24 h of growth at 37°C) with a sterile tip and spread a thin film on one spot of the "target plate"; the microbial film is then coated with a matrix (typically 1-1.5 µL of CHCA in standard OS solution (500 µL acetonitrile + 475 µL Water mQ + 25 µL trifluoroacetic acid) for the Bruker instrument), which can act as a proton source for ionization of the analyte under test. In case the identification yield is to be increased, 70% Formic Acid can be added before the addition of the matrix, while in case the direct application procedure does not work, a protein extraction step with ethanol-acetonitrile can be performed. The sample-matrix mixture is allowed to dry at room temperature for about 10 minutes and then introduced into MALDI-TOF for data acquisition, which is processed by the software, and the spectra are compared with reference libraries (Croxatto et al., 2011). When the target plate is placed inside the mass spectrometer, it is bombarded at several points per spot by short laser pulses, usually nitrogen (Croxatto et al., 2011); the laser frequency is low and ranges from 10 Hz to 1 KHz (Figure 7).

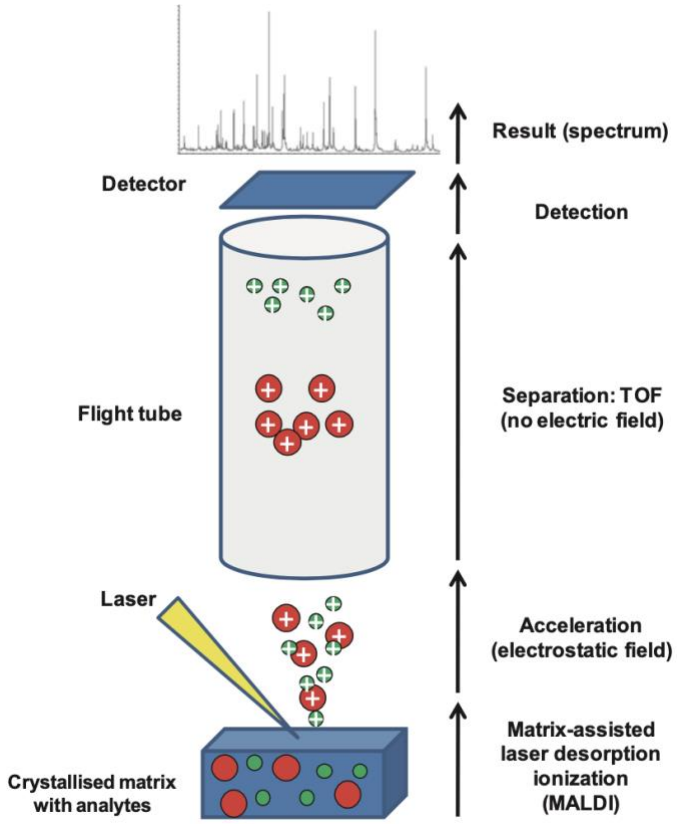

Figure 7: Technical description of MALDI-TOF MS (Reproduced from Croxatto et al., 2011)

The matrix protects the molecules from the laser, preventing them from being hit directly and facilitating vaporization and ionization. On the target plate, in addition to the samples to be analyzed, Bacterial Test Standard (BTS) as control is also deposited and undergoes the same treatment (Brunelli, 2016). Laser irradiation results in vibrational excitation of the matrix and ejection (desorption) of analyte molecules surrounded by groups of matrix molecules, water, and ions. Subsequently, the matrix molecules transfer protons to the analyte, and thus positively charged analyte cations occur in the gas phase. These are then accelerated through an electric field inside the ionization chamber at a rate that depends on the m/z ratio. Next, the particles enter the analyzer arriving at the detector, which measures their "travel time" (time of flight). Based on the time of flight, the m/z ratio of each particle can be determined, and from there, a mass spectrum of the analyzed sample is generated (Brunelli, 2016). Smaller, charged ions travel faster than larger, less charged ions;, the TOF required to reach the detector depends on the sample's mass (m) and charge (z) and is proportional to the square root of m/z. The mass spectrum formed depends on both the m/z and the intensity of the ions (no. ions with specific m/z that reached the detector), and the result consists of peaks ranging from 1000 to 20,000 m/z. The resulting spectrum is then compared with a database for species or genus identification (Croxatto et al., 2011). The resulting data are collected and processed by special software that generates peptide spectra and characteristic profiles of

bacterial cells; each microorganism has its specific spectrum ("fingerprint spectrum") with different peaks corresponding to soluble proteins present in high amounts, such as ribosomal and heat-shock proteins. The spectrum is compared to identify the sample under analysis using specific algorithms, with known spectra contained within databases (reference strains or strains identified with high confidence) (Brunelli, 2016). In summary, the MALDI-TOF mass spectrometer identifies bacteria using the mass spectrum of highly abundant proteins, mainly ribosomal proteins, which generate a "fingerprint" typical of the bacterium. This "fingerprint" is used to query a database, the spectra "library," to find its perfect match. Species identification by MALDI-TOF has very high reliability, but it is essential to have a good database. In fact, if the spectrum is not in the library, identification is not possible, so the completeness and quality of the library are crucial for better identification (Croxatto et al., 2011). Data acquisition is controlled using Maldi Bio-typer (MBT) Compass IVD software: the spectrum of the sample under analysis is transformed into a list of peaks, which is compared with lists of peaks of reference organisms in the reference library database using a biostatistics algorithm and finally, a log (score) between 0.000 and 3.0000 is generated. The higher the log, the greater the degree of similarity between the pattern of unknown peaks and the list of peaks in the reference library database. A log greater than or equal to 2.000 is considered acceptable for species-level identification, while in the case of a log less than 2.000, samples can be processed with an alternative sample procedure and the analysis repeated. The log ranges defined by MBT Compass IVD reflect the probability of organism identification; the results should be reviewed by a clinical microbiologist, and the final identification should be based on all available relevant information [BRUKER© MALDI BioTyper brochure, RUO (https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/maldi-biotyper-library-ruo.html)] (Figure 8).

Range	Description	Symbols	Color
2.300 ... 3.000	highly probable species identification	(+++)	green
2.000 ... 2.299	secure genus identification, probable species identification	(++)	green
1.700 ... 1.999	probable genus identification	(+)	yellow
0.000 ... 1.699	not reliable identification	(-)	red

Figure 8: Log score ranges MALDI Biotyper Compass IVD
(Reproduced from https://www3.ha.org.hk/haconvention/hac2015/proceedings/downloads/PS3.6.pdf)

One problem in using MALDI-TOF is the reproducibility of the spectra. These can be different within the same bacterial species depending on the strain or the conditions under which the bacterium has multiplied; reproducibility depends on several factors, including the instrument, the matrix used, the

age of the colony, the sample/matrix ratio, the sample concentration, the culture medium, and the growth conditions. Several studies have shown that peaks are also present that are conserved under different experimental conditions, including ribosomal proteins, which can be used as biomarkers. To improve reproducibility, diagnostic laboratories need to establish a standard sample preparation, from matrix choice to concentrations and crystallization conditions (Croxatto et al., 2011). In general, excellent results have been demonstrated for bacterial identification by MALDI-TOF MS: many studies have shown that most bacterial groups (*Enterobacteriaceae*, Gram-negative nonfermenting bacteria, staphylococci, and hemolytic streptococci) have been correctly identified at the species level (Seng et al., 2009), and for staphylococci, the use of MALDI-TOF MS has brought the great advantage of quickly recognizing *S. aureus* and some NAS (Croxatto et al., 2011). When using MALDI-TOF MS, appropriate maintenance and quality control in identification are crucial. Performance can be checked by testing known bacterial strains whose spectra are available in the database; these routine internal quality checks can be done on *E. coli* ATCC 25922 or *S. aureus* ATCC 25923, and retained peaks with intensities above 200 are defined. If the results are compromised by technical problems or poor maintenance, the instrument can be recalibrated using BTS to identify and resolve them; this consists of lyophilized extracts of *E. coli* and two additional proteins (RNAse A and myoglobin), which show peaks at 13 683 and 16 952 m/z, respectively (Croxatto et al., 2011). Other problems may result from inadequate sample deposition on the target, poor cleaning of the target, and the cleanliness of the laser source. Several studies have compared the performance of MALDI-TOF MS with conventional methods and have shown that this technique has high accuracy for most microbial identifications and is performed the same or better than conventional techniques. The most obvious differences are observed in sample identification's estimated time and cost. The cost of bacterial identification by MALDI-TOF MS has been estimated to be only 17-32% (about € 1.43/sample) of the costs of conventional identification methods (about € 4.6-8.23/sample) (Seng et al. 2009). The instrument's purchase price is high, but the operating costs are considerably lower than those of conventional identification methods. MALDI-TOF MS allows a significant gain in both technician's work time (pre-analytical procedure to prepare samples) and delivery time (automated analytical procedure to obtain results). The time required for bacterial identification from intact cells is 6-8.5 min compared to 5-48 h for conventional identification, as estimated by Seng et al. (2009), and the time advantage is even more pronounced when multiple isolates are analyzed in parallel (Croxatto et al., 2011). Another advantage of using MALDI-TOF MS is related to the problem of the increase in multidrug-resistant organisms and the increased need to provide appropriate and rapid antimicrobial therapies; using conventional identification methods takes a long time to identify the optimal therapy, whereas using this innovative tool reduces the

organism identification time by 1.2-1.5 days, thus allowing for a more immediate response (Beganovic et al., 2017; Vlek et al., 2012).

Polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP)

The polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) technique is based on the digestion of PCR amplicons with appropriate restriction enzymes to have distinct polymorphic fragments used as markers for species identification (Kim et al., 2017). PCR-RFLP is a fast and easy way to detect codon mutations based on an alternative restriction enzyme (RE) site caused by a codon mutation (Zheng et al., 2015). The PCR-RFLP designed by Botstein et al. is also known as a cleaved amplified polymorphic sequence. In this technique, a PCR amplicon is treated by a certain RE that cuts the DNA in a unique restriction site, called the recognition site, to produce several DNA fragments in different sizes (Hashim et al., 2019). The digested amplicons are then loaded into a gel, and an electric field is applied where the differently sized bands will move at variable distances across the gel (Panneerchelvam et al., 2003). PCR-RFLP identifies minor variations in a gene where a single-base substitution creates or abolishes a recognition site for the RE (Hashim and Al- Shuhaib, 2019). The most potent aspect of PCR-RFLP is its ease because it can be performed without significant experience in molecular biology. Despite the simplicity of use and extreme ease of PCR-RFLP, it is limited only to the recognition site of RE, and other sequences are ignored unless double digestion is used with another RE.

Consequently, the main limitations of PCR-RFLP are the necessity for specific RE and the difficulty of identifying the exact variation if several single nucleotide polymorphisms are being targeted simultaneously (Hashim et al., 2019). Mixing two enzymes in one reaction can partially solve this problem (Shirasawa et al., 2016). Regarding digestion, there are additional complications because of the different types of cofactors and the concentrations needed for each RE to undertake its scheduled task of standardized digestion (Rasmussen et al., 2011). Additionally the higher costs of PCR-RFLP resulting from the higher costs of double or triple digestion add another inevitable limitation (Hashim et al., 2019).

The gut microbiome

The intestinal tract of mammals is colonized by a large population of microorganisms, including bacteria that live in symbiosis with the entire organism and colonizes specific tracts. This population is called the intestinal microbiota and varies in composition depending on many factors, including

the animal's diet, the substrate derived from it, and the pH present in the intestinal tract. Until recently, the microbiota was defined as "the collection of multiple species of microorganisms living in a particular ecological niche, such as soil, seawater, or an animal's digestive system, interacting with each other, the surrounding environment, and their eventual host; it is a complex system, the structure of which is continually influenced by variation in chemical, physical, and biological factors" (Little et al., 2008; Konopka et al., 2009); while the microbiome was defined as "the complex of genomes of all microorganisms present in a microbiota" (Cryan et al., 2012; Ursell et al., 2012). According to a proposal by Marchesi and Ravel in an editorial in the journal "Microbiome," these terms take on a somewhat more articulated role. The term microbiota defines the set of microorganisms present in an environment, but these are described and defined using molecular methods that rely primarily on the analysis of 16s rRNA genes, 18s rRNA genes, or other marker genes or genomic regions. Taxonomic levels of the microbiota are assigned using various tools that assign a "microbial taxon" (bacteria, archaea, or lower eukaryotes) to each sequence at different taxonomic levels ranging from phylum to species.

The microbiome is defined as the habitat in which microorganisms (bacteria, archaea, eukaryotes, and viruses) live, as well as their genes and surrounding environmental conditions. As reported in the editorial, "this definition is based on that of biome, i.e., the biotic and abiotic factors of given environments." Others in the sector limit the definition of the microbiome to all genes and genomes belonging to a microbiota. Instead, it is argued that it is the definition of the metagenome, which in combination with the environment, constitutes the microbiome (Marchesi and Ravel, 2015).

The indices of diversity: Alpha- and Beta-diversity

Biodiversity is the earth's variety of plant, animal, or microbial species. The unit used to define biodiversity is the species, which is the set of individuals able to exchange genetic material with each other that will be passed on to future generations. Biodiversity can be measured on multiple levels, such as population or community, and can be distinguished into three different components (Walters et al., 2020):

- Alpha Diversity: diversity that considers the number of species present in a small, uniform area, such as the fecal microbiota of an animal;
- Beta Diversity: diversity that describes how diversity varies from one habitat to another, such as the gut microbiota changing from one animal to another;
- Gamma Diversity: diversity that considers the species present in a region, defined as an area that does not include significant barriers to the dispersal of organisms.

Multiple methods have been used to quantify biological diversity. Two main approaches have been used to measure the diversity of a sample, richness (richness) and evenness (evenness). The concept of richness defines the number of different types of organisms present in a community; "species richness" then defines the number of species present in each biological niche. Species richness does not consider the number of individuals belonging to each species and diversity depends not only on richness but also on evenness (evenness). Evenness is associated with the uniformity of the different species sizes. Evenness measures the relative abundance of the different species that make up a community (Figure 9). Generally, when richness and evenness increase, diversity also increases (Bo-Ra et al., 2017).

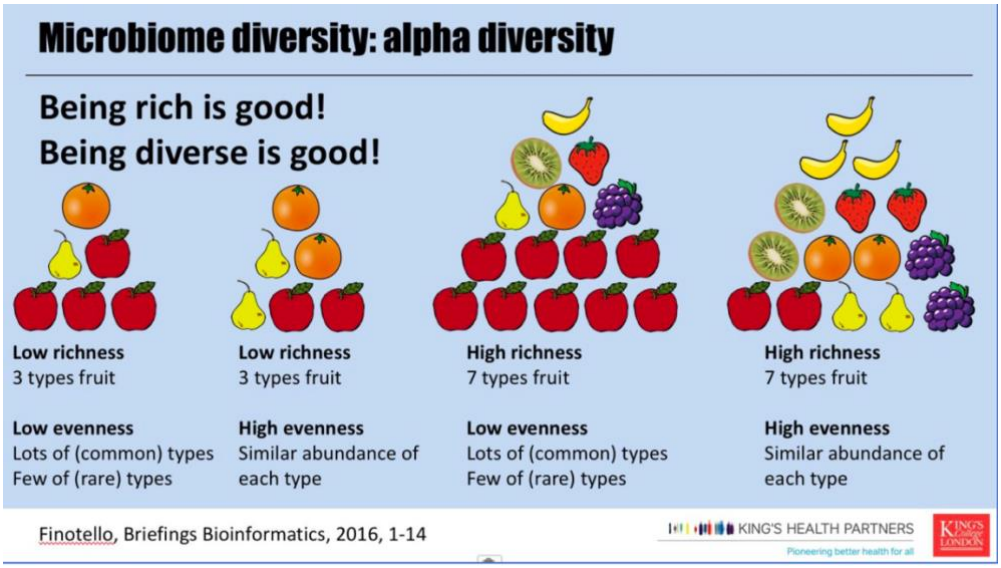

Figure 9: Alpha diversity metrics can look at the richness, evenness, or both within a sample (Reproduced from conference presentation Finotello et al., 2016)

Below are some statistical indices used to quantify biodiversity (Bo-Ra et al., 2017; Colla et al., 2014):

- Observed OTUs: Index of diversity based on the number of operational taxonomic units (OTUs) observed in each sample. An OTU is simply the group of organisms that are being studied at a given time; it is an index of richness;
- Observed Species: Diversity index that is based on the richness in terms of species present in a sample;
- Chao1: Richness index that is based on abundance, i.e., to the number of individuals belonging to a given class in a sample, giving greater weight to low abundance species;
- ACE: Non-parametric diversity index that is used to estimate the number of species and thus the richness (in the sense of species density) of a given sample, uses highly abundant species (presence/absence) and low abundant species (exact frequencies);

- Fisher Alpha: Diversity index that describes the relationship between the number of species and the number of individuals of that species;
- Shannon: Diversity index that considers both richness and evenness of the species present, giving greater weight to the former;
- Simpson: Diversity index that considers both richness and evenness of the species present, giving greater weight to the latter;
- Equitability: Index of diversity that expresses the degree of homogeneity with which individual microorganisms are distributed among the various species that make up a community.

Methods for studying the microbiome

To define a microbial community, it is important to dwell on the level of investigation:
- Molecular;
- Cellular;
- Multicellular (organism);
- Population;
- Community.

Each level, in general, depends on those below, but any level takes on characteristics that cannot be deduced from the analysis of individual components since they are due to the various levels' interactions. These characteristics are biologically essential for the functioning of the system. For example, certain organic substances are degraded by different microbial populations where individual species alone cannot carry out the entire degradation process. Specific analytical tools are needed to investigate each level of biological complexity. Yet even though microbial species are of considerable importance, long-time studies were based on laboratory methods that depended on bacterial cultivation. At the same time, it is known that most environmental microorganisms cannot be cultivated by traditional methods usually used in the laboratory. Culture-independent molecular techniques have been developed and applied to study microbial diversity. These new methods have complemented classical methods, such as plate culture or analysis of the metabolic characteristics of microbial isolates in culture, enabling greater awareness of microbial diversity. In general, techniques for analyzing microbial communities can be classified into:
- Conventional methods, which analyze the phenotypic characteristics of microorganisms through techniques based on their cultivation, such as enrichment for specific metabolic activities or isolation based on selective culture media;
- Molecular methods, which allow the analysis of genetic characteristics.

Specifically, speaking of genetic studies, we resort to the analysis of specific genetic "markers" that allow us to distinguish the various individuals in the communities taxonomically or will enable us to highlight the presence of metabolic activities. In this regard, it is possible to divide these techniques into two groups, namely:

- Techniques based on amplification by PCR;
- Biochemical techniques, which are independent of amplification by PCR.

Amplification-based techniques include analyzing the microbiota by amplifying and sequencing 16S DNA (Fakruddin et al., 2013; Sarangi et al., 2019).

16S DNA sequencing for the study of the microbiota

The 16S rRNA gene is a gene consisting of ten conserved and nine hypervariable regions that are present in all bacteria (Figure 10). Because of the restrictions to which the structure of rRNA is subjected, as a consequence of the fact that it must assume a defined secondary structure and must interact with several proteins to form a functional ribosome, the rate of sequence variation of genes encoding for rRNA is far lower than that of other genes (Woese et al., 1977 and Dallago et al., 2015). Consequently, it is possible to identify phylogenetic relationships over huge evolutionary distances. This type of gene functions as a molecular clock and allows accurate determinations of phylogenetic distances. The information contained in rRNAs (such as the 16S rRNA) means that sequencing the genes (rDNAs) coding for these macromolecules allows genetically based identification of genera and species, even in the case of bacteria that are difficult to classify. With these procedures and molecular sequencing, it is possible to study different bacterial communities in different habitats. This technique allows us to simultaneously analyze a community of microorganisms and identify a single microorganism that is part of the community. In this way, it is possible to study the taxonomy of the microbiota and hypothesize its functions (Woese et al., 1977).

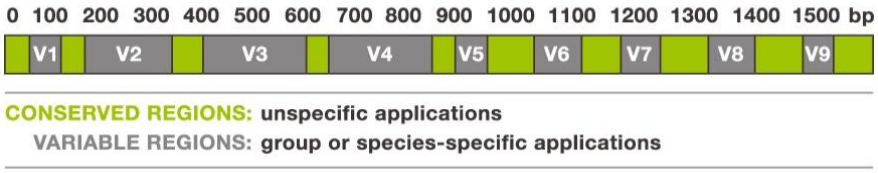

Figure 10: Schematic diagram of the subdivision of hypervariable regions within the 16S rRNA gene sequence (Reproduced from https://biology.stackexchange.com/questions/54823/what-causes-the-variable-conserved-structure-in-the-16s-rrna-gene)

The intestinal microbiota of the calf

The gut microbiota plays a fundamental role in every animal's life; it allows it to contribute to the state of well-being, ensuring the perfect functioning of the digestive system. Diet is one of the main

factors influencing the composition of the gut microbiota and can also play an important role in changes observed over time in the ruminal microbiome of newborn calves (Malmuthuge N. et al., 2015). The microbiota contributes to the proper functioning and health of the gastrointestinal tract by influencing nutrient absorption, the efficiency of nutrient utilization, and the subsequent growth of animals (Malmuthuge N. et al., 2017).

Microbial colonization of the gut

The gastrointestinal tract development in neonatal humans and animals is a dynamic process influenced by many factors such as genetics, environmental, nutritional and the concomitant development of the intestinal microbial communities (Figure 11). This is also accurate for ruminants, where the first month of life is more challenging as the rumen is less developed (Amin et al., 2021).

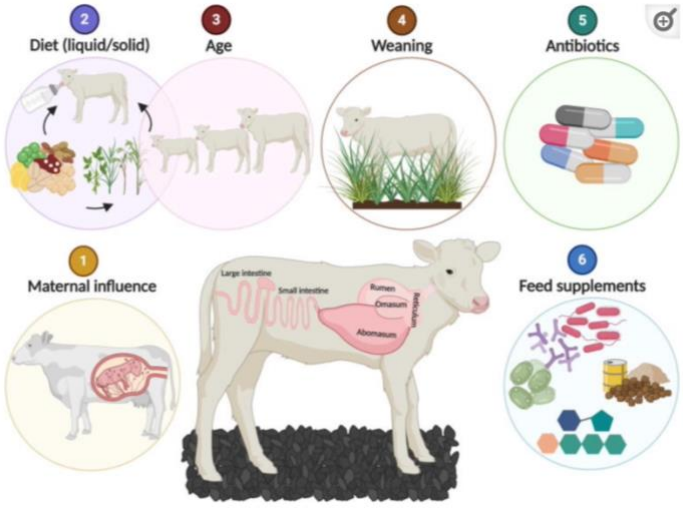

Figure 11: Factors that influence the initial establishment and development of microbial communities throughout the gastrointestinal of neonatal calves (Reproduced from Amin et al., 2021)

At birth, the intestinal tract of the calves is almost empty of bacteria, but due to colonization by environmental bacteria, its microbial population begins to enrich within a short time. During the first few days and weeks, as the calf feeds, the esophageal groove causes most of the milk to seep directly into the abomasum, thus ensuring effective digestion and absorption of nutrients (Church, 1988). Not all milk ends up in the abomasum; in fact, some of it flows into the rumen, which is still developing, providing nutrients for the microorganisms in it (Fonty et al., 1987; Morvan et al., 1994). Calves are born with a rumen that is physiologically unable to function. During a few weeks, in answer to increased ingestion of solid food and increased fermentation of these by microorganisms, the rumen becomes functional (Heinrichs, 2005). The rumen is a bacterial-rich organ; several studies have shown how the microbial communities that develop in the first days of life determine the subsequent

communities present in the adult cattle rumen, thus positively or negatively altering animal productivity and health (Yanez-Ruiz et al., 2015). Early studies carried out on the microbial communities that develop in the rumen during the neonatal period showed that initially, there is rapid colonization of the ruminal walls by anaerobic-facultative microbial taxa, which gradually decrease between 6 and 8 weeks of age, being replaced by exclusively anaerobic taxa after eight weeks of age (Bryant et al., 1962; Fonty et al., 1987; Minato et al., 1992). One of the main factors that can alter the composition of the microbiota of these animals is the diet (Malmuthuge et al., 2015). Little is known about the microbiota present beyond the ruminal level in cattle. The study of the composition of the microbiota present in the feces of calves has shown how microbial diversity increases with increasing days and undergoes substantial changes during the pre-weaning period (Edrington et al., 2012).
A healthy gut microbiota prevents the colonization of foreign pathogens and improves host immune systems through interactions between antigens and immune cells during the early stage of life. The increased intestinal permeability and disturbance of gut microbiota are key factors leading to pathogen-induced diarrhea. Calf diarrhea association with both functional dysbiosis and microbial composition has shed light on diarrhea with microbial modulation of gut ecosystems (Fan et al., 2021).

Microbiota and evaluation of other aspects related to dairy cattle

A potential role in developing dysbiosis of gut microbiota and AMR in animal husbandry could be attributable to waste milk (WM), particularly antibiotic-containing WM, as it could act on the intestinal flora of calves by selecting forms with AMR. According to a recent study, by feeding a group of calves with WM containing antimicrobial residues, they develop *E. coli* AMR strains in the intestine (Duse et al., 2015). If stably present in the intestinal microbiota, these forms of AMR may persist in the herd, creating an environmental shedding reservoir. Based on the scientific literature, it is likely that the antimicrobial residues present in WM provide a unique selective environment. This environment could lead to a growth in the number of resistant bacteria and the exchange of genetic material between bacteria by conjugation or transformation (Ricci et al., 2017). It is reasonable to consider the hypothesis that feeding derived from WM contaminated with antimicrobial-resistant bacteria (pathogenic or commensal) or contaminated with AMR-encoding genes may increase the risk of the rate of presence of resistant bacteria in the calf.
It would also appear that the young age of the animal has a positive correlation with the elimination of AMR bacteria. Several studies have shown that feeding WM containing antimicrobial residues in 2- to 3-week-old calves increases the elimination of *E. coli* AMR through feces. This trend would appear to decrease as calf age increases (Aust et al., 2013; Brunton et al., 2014; Pereira et al., 2014).

One study confirmed that after weaning at 60 days of age, the elimination of resistant *E. coli* drops dramatically to the point of showing no significant differences between animals fed WM and the control group (Brunton et al., 2014). In addition to pathogens, beneficial commensal bacteria that serve as reservoirs for AMR genes are also present among AMR-affected bacteria: these types of microorganisms harbor the so-called resistome (D'Costa et al., 2006; Martinez et al., 2015; Surette and Wright, 2017; Woolhouse and Ward, 2013) (Figure 12). This reservoir resides in a group of microorganisms of different phylogenetic origins in which horizontal gene transfer occurs (Shterzer and Mizrahi, 2015). Generally, mobile elements are involved in gene transfer with AMRs, thus suggesting that the commensal microbial community may act as a reservoir of AMRs, not only for intestinal pathogenic bacteria but also for environmental bacteria through feces. Anaerobic ruminal bacteria possess intrinsic resistance to certain antimicrobials (Fulghum et al., 1968). Gram-positive ruminal bacteria are much more sensitive to antimicrobials used as growth promoters (Nagaraja and Taylor, 1987). Confirming this, a positive correlation has been observed between feeding the calf with WM and colostrum containing antimicrobial residue and the increase of bacteria with AMR in the feces (Berge et al., 2006; Thames et al., 2012; Aust et al., 2013; Brunton et al., 2014; Pereira et al., 2014). There are essentially three possible causes of this correlation:

- The calf takes on resistant bacteria that are already present in colostrum or WM;
- Antimicrobial residues in milk may select resistant bacteria (e.g.: Extended Spectrum Beta Lactamase (ESBL) - producing bacteria with plasmid localization);
- Antimicrobial residues in milk can create de novo AMR by selecting spontaneous mutant strains.

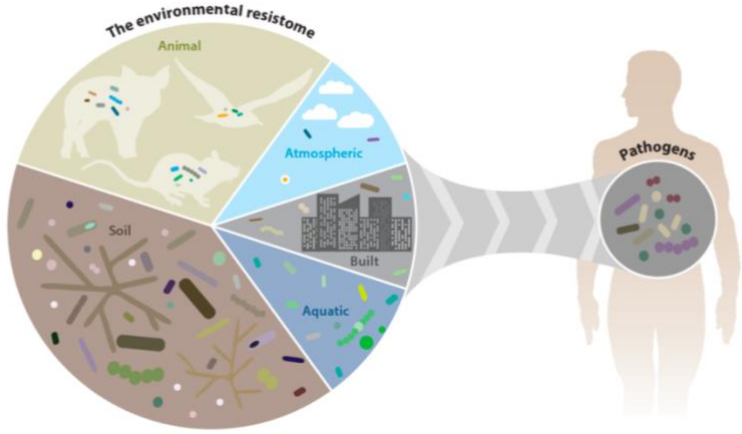

Figure 12: Schematic illustration of the contribution of environmental resistome to animal and human resistome (Reproduced from Surette et Wright, 2017)

On the other hand, other studies have shown that the feeding of calves with raw WM had a more significant average daily gain and a higher concentration of total whey protein in relation to standard raw milk (Zou et al., 2017). However, antibiotic residues and the presence of some bacterial pathogens may limit the use of raw WM as a source of feed in calves because this could spread

antibiotic-resistant bacteria into their feces. Properly pasteurized WM feeding can reduce the bacterial load in milk and the risk of disease in calves. Nevertheless, it is advisable to avoid feeding raw WM obtained from cows treated with antibiotics for a long-time during lactation (Ma et al., 2022). Maintaining a healthy gastrointestinal microbiota is essential for ruminant health and productivity. To prevent infections such as metritis or mastitis (Vasquez et al., 2016), the administration of antibiotics in cows is widely adopted, and these can disrupt the indigenous microbiota and increase antibiotic-resistant genes in dairy cows (Wichmann et al., 2014; Liu et al., 2016). After antibiotic administration, ecological disorders in the microbiota may persist for long periods, and some taxa of indigenous bacteria may not be recovered (Jernberg et al., 2010; Nobel et al., 2015; Korpela et al., 2016). Related to intestinal inflammation (Cross et al., 2016), behavioral responses, and alternating stress (Daghrir et al., 2013; Martin, 2011) is the microbiota-gut-brain axis, bidirectional communication between neural, hormonal, and immunological pathways (Ben Saad et al., 2016). This two-way communication could direct the activities of brain function (a stress-related hormone) with the immune response through the activities of the intestinal microbiota and, consequently, the behavioral response. The gastrointestinal microbiota is called the "second genome" and plays an important role in animal growth and health, especially in ruminants. Studies have shown that gastrointestinal microbes can influence body weight and digestion and decrease the risks of infection and autoimmune diseases. The gastrointestinal microbiome is a complex ecosystem composed of various types of microorganisms (bacteria, archaea, protozoa, fungi) interacting with each other; it is involved in the regulation of different physiological parameters of the host, including milk yield, the digestive capacity of nutrients such as starch or pectin, and methane production (Chen et al., 2020) (Figure 13).

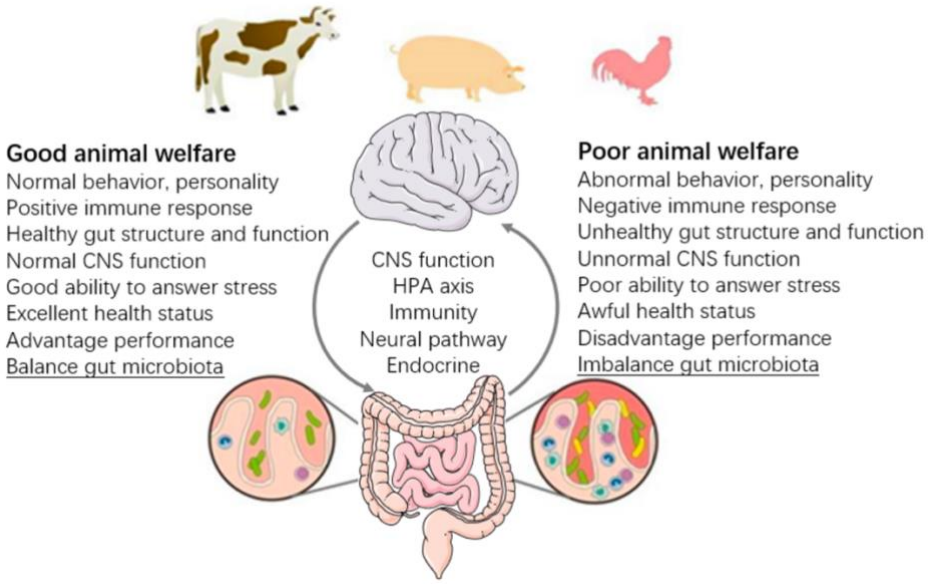

Figure 11: Animal welfare in the implication and perspective of the gut microbiome (Reproduced from Chen et al., 2020)

The incidence of mastitis is associated with reduced diversity and a change in intramammary microbiota composition (dysbiosis). But whether microbial dysbiosis is the cause or consequence of infectious mastitis remains a debate. The growing interest in developing new drugs to treat bovine mastitis dictates the need to better understand the various factors that can influence mammary homeostasis. The commensal microbiota inhabiting multiple niches in the udder, including the teat apex, teat canal, and intramammary ecosystem, can modulate the cow's susceptibility to IMI by mastitis pathogens through direct microbe-microbe cross-talk, indirect stimulation of immunity, or both. Although ongoing, current understanding of the udder microbial ecosystem suggests that optimal diversity of the intramammary microbiota, composed of a healthy balance of commensal and opportunistic bacterial groups, is essential to maintain a balance between pro- and anti-inflammatory responses and thus to maintain mammary homeostasis. Cow physiology, udder anatomy, genetic traits associated with immune reactivity, and environmental factors can alter the composition of the udder microbiota. Whether various profiles of the udder microbiota may confer resistance to IMI by mastitis pathogens remains poorly known. Much effort is needed to identify the potential mechanisms by which the commensal microbiota of various udder compartments interact with each other, with mastitis pathogens, and with the immune system (Derakhshani et al., 2018) (Figure 14).

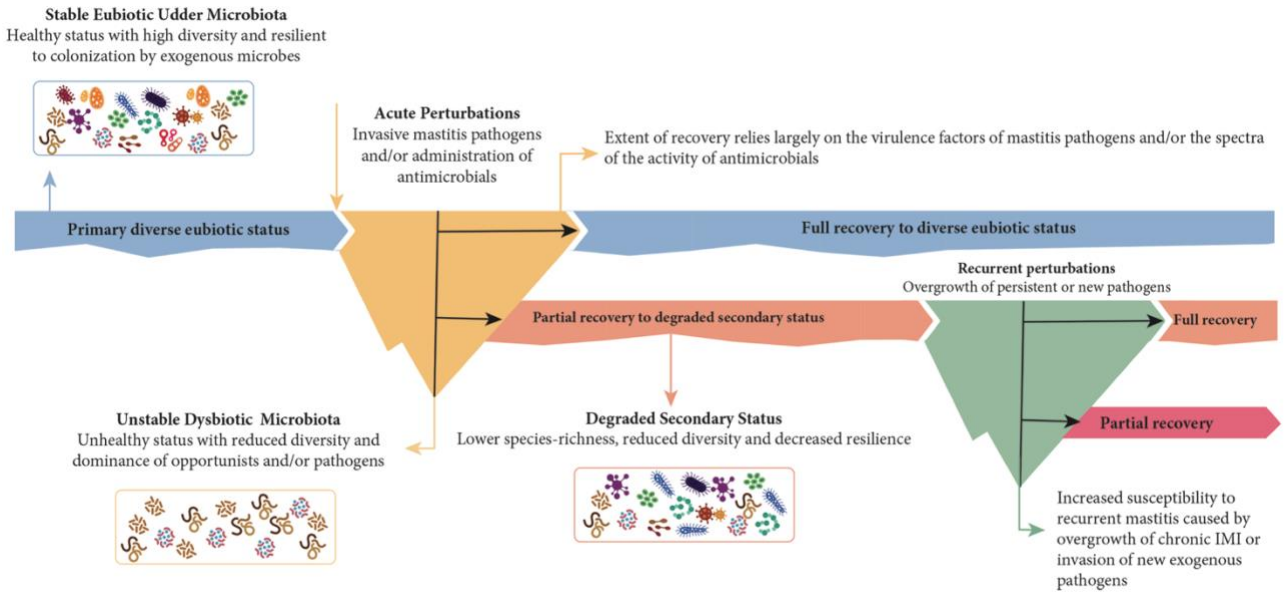

Figure 14: Potential role of microbiota dysbiosis in modulating mastitis susceptibility (Reproduced from Derakhshani et al., 2018)

Bibliography

Introduction

Addis M.F., G. M.G. Puggioni; C. Cacciotto; V. Tedde; D. Pagnozzi; S. Uzzau; V. Bronzo; A. Casula; G. Curone; P. Moroni; C. Locatelli (2017) Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis. JDS VOLUME 100, ISSUE 4, P2944-2953, APRIL 01. doi: 10.3168/jds.2016-12110. Epub 2017 Jan 26. PMID: 28131570

Addis M.F. (2017) Markers and immunoassays for detecting mastitis Scientific School: Understanding and Controlling Dairy Ruminant Mastitis. Tramariglio, Alghero, September 5-7, 2017

Addis M.F., Tedde V., Puggioni G.M.G., Pisanu S., Casula A., Locatelli C., Rota N., Bronzo V., Moroni P., Uzzau S. (2016) Evaluation of milk cathelicidin for detection of bovine mastitis. Journal of Dairy Science, vol. 99, pp. 8250–8258. doi: 10.3168/jds.2016-11407. Epub 2016 Aug 10. PMID: 27522416

Adlerova, L., Bartoskova, A., and Faldyna, M. (2008) Lactoferrin: a review, Vet. Med., 53, 457-468,doi: 10.17221/1978-VETMED

Aghamohammadi M., Haine D., Kelton D.F., Barkema H.W., Hogeveen H., Keefe G.P., Dufour S. (2018) Herd-Level Mastitis-Associated Costs on Canadian Dairy Farms. Front Vet Sci. May 14;5:100. doi: 10.3389/fvets.2018.00100. PMID: 29868620; PMCID: PMC5961536

Agier J., Efenberger M., and Błaszczyk E.B. (2015) Cathelicidin impact on inflammatory cells. Cent Eur J Immunol; 40(2): 225–235. doi: 10.5114/ceji.2015.51359. Epub 2015 Aug 3. PMID: 26557038; PMCID: PMC4637384

Alhussien M.F., Dang A.K. (2018) Milk somatic cells, factors influencing their release, future prospects, and practical utility in dairy animals: An overview. Veterinary World, vol. 11(5), pp. 562–577. doi: 10.14202/vetworld.2018.562-577. Epub 2018 May 2. PMID: 29915493; PMCID: PMC5993762

Amin N, Seifert J. (2021) Dynamic progression of the calf's microbiome and its influence on host health. Comput Struct Biotechnol J. Jan 26;19:989-1001. doi: 10.1016/j.csbj.2021.01.035. PMID: 33613865; PMCID: PMC7868804

Azooz MF, El-Wakeel SA, Yousef HM. (2020) Financial and economic analyses of the impact of cattle mastitis on the profitability of Egyptian dairy farms. Vet World. 2020 Sep;13(9):1750-1759. doi: 10.14202/vetworld.2020.1750-1759. Epub 2020 Sep 2. PMID: 33132585; PMCID: PMC7566233

Aust V., Knappstein K., Kunz H.J., Kaspar H., Wallmann J. and Kaske M. (2013) Feeding untreated and pasteurized waste milk and bulk milk to calves: effects on calf performance, health status and antibiotic resistance of faecal bacteria. Journal of Animal Physiology and Animal Nutrition, 97, 1091–1103. doi: 10.1111/jpn.12019. Epub 2012 Dec 4. PMID: 23205592

Bar D., Tauer L.W., Bennett G., González R.N., Hertl J.A., Schukken Y.H., Schulte H.F., Welcome F.L., Gröhn Y.T. (2008) The cost of generic clinical mastitis in dairy cows as estimated by using dynamic programming. J Dairy Sci. 2008 Jun;91(6):2205-14. doi: 10.3168/jds.2007-0573. PMID: 18487643

Beganovic M., Costello M., Wieczorkiewicza S. M. (2017) Effect of Matrix-Assisted Laser Desorption Ionization–Time of Flight Mass Spectrometry (MALDI-TOF MS) Alone versus MALDI-TOF MS Combined with Real-Time Antimicrobial Stewardship Interventions on Time to Optimal Antimicrobial Therapy in Patients with Positive Blood Cultures. Journal of Clinical Microbiology; Volume 55 Issue 5. doi: 10.1128/JCM.02245-16. Epub 2017 Feb 22. PMID: 28228491; PMCID: PMC5405261

Benić M., Maćešić N., Cvetnić L., Habrun B., Cvetnić Z., Turk R., Đuričić D., Lojkić M., Dobranić V., Valpotić H., Grizelj J., Gračner D., Grbavac J., Samardžij M. (2018) Bovine mastitis: a persistent and evolving problem requiring novel approaches for its control - a review. Veterinarski arhiv, 88 (4), 535- 557, 2018. doi: 10.24099/vet.arhiv.0116

Ben Saad H, Kharrat N, Krayem N, Boudawara O, Boudawara T, Zeghal N, et al. (2016) Biological properties of Alsidium corallinum and its potential protective effects against damage caused by potassium bromate in the mouse liver. Environ Sci Pollut Res Int. 23:3809–23. doi: 10.1007/s11356-015-5620-2. Epub 2015 Oct 24. PMID: 26498820

Berge A.C.B., Moore D.A. and Sischo W.M. (2006) Field trial evaluating the influence of prophylactic and therapeutic antimicrobial administration on antimicrobial resistance of fecal Escherichia coli in dairy calves. Applied and Environmental Microbiology, 72, 3872–3878. doi: 10.1128/AEM.02239-05. PMID: 16751491; PMCID: PMC1489621

Berry D.P., Meaney W.J. (2006) Interdependence and distribution of subclinical mastitis and intramammary infection among udder quarters in dairy cattle. Prev Vet Med. 2006 Jul 17;75(1-2):81-91. doi: 10.1016/j.prevetmed.2006.02.001. Epub 2006 Mar 23. PMID: 16563533

Bizzini A, Jaton K., Romo D., Bille J., Prod'hom G. & Greub G. (2011) Matrix-assisted laser desorption ionization-time of flight mass spectrometry as an alternative to 16S rRNA gene sequencing for identification of difficult-to-identify bacterial strains. J Clin Microbiol 49:

693–696. doi: 10.1128/JCM.01463-10. Epub 2010 Nov 24. PMID: 21106794; PMCID: PMC3043501

Bo-Ra K., Jiwon S., Robin B.G., Jun H.L., Doo W.K., Kuk-Hwan S., Ju-Hoon L., Hyeun B. K., Isaacson R. (2017) Deciphering Diversity Indices for a Better Understanding of Microbial Communities. Journal of Microbiology and Biotechnology, 27, 2089– 2093. doi: 10.4014/jmb.1709.09027. PMID: 29032640

Botstein D, White RL, Skolnick M, Davis RW. (2019) Construction of a genetic linkage map in man using restriction fragment length polymorphisms. Am J Hum Genet. 1980 May;32(3):314-31. PMID: 6247908; PMCID: PMC1686077

Braem G, De Vliegher S, Verbist B, Piessens V, Van Coillie E, De Vuyst L, et al. (2013) Unraveling the microbiota of teat apices of clinically healthy lactating dairy cows, with special emphasis on coagulase-negative staphylococci. J Dairy Sci. 96:1499–510. doi: 10.3168/jds.2012-5493. Epub 2013 Jan 10. PMID: 23313004

Brock JH. (2002) The physiology of lactoferrin. Biochem Cell Biol. 2002;80(1):1-6. doi: 10.1139/o01-212. PMID: 11908632

Bronzo V., Lopreiato V., Riva F., Amadori M., Curone G., Addis M.F., Cremonesi P., Moroni P., Trevisi E., Castiglioni B. (2020) The role of innate immune response and microbiome in resilience of dairy cattle to disease: the mastitis model. Animals (Basel), vol. 10(8), 2020, p. 1397. doi: 10.3390/ani10081397. PMID: 32796642; PMCID: PMC7459693

BRUKER© MALDI BioTyper brochure, RUO (https://www.bruker.com/en/products-and-solutions/microbiology-and-diagnostics/microbial-identification/maldi-BioTyper-library-ruo.html)

Brunelli T. (2016) Uso della spettrometria di massa MALDI-TOF per l'identificazione di Micobatteri Non Tubercolari. Tesi di Specializzazione

Brunton L.A., Reeves H.E., Snow L.C. and Jones J.R. (2014) A longitudinal field trial assessing the impact of feeding waste milk containing antibiotic residues on the prevalence of ESBL-producing Escherichia coli in calves. Preventive Veterinary Medicine, 117, 403–412. doi: 10.1016/j.prevetmed.2014.08.005. Epub 2014 Aug 14. PMID: 25172121

Bryant M.P., Robinson I.M. (1962). Some nutritional characteristics of predominant culturable ruminal bacteria, Journal of Bacteriology. doi: 10.1128/jb.84.4.605-614.1962. PMID: 14016429; PMCID: PMC277935

Cantekin, Z., Ergün, Y., Dogruer, G., Sarıbay, M.K., & Solmaz, H. (2015). Comparison of PCR and culture methods for diagnosis of subclinical mastitis in dairy cattle. Kafkas Universitesi Veteriner Fakultesi Dergisi, 21, 277-282

Ceciliani F, Ceron JJ, Eckersall PD, Sauerwein H. (2012) Acute phase proteins in ruminants. J Proteomics. 2012 Jul 19;75(14):4207-31. doi: 10.1016/j.jprot.2012.04.004. Epub 2012 Apr 11. PMID: 22521269

Cembrola G. (2005) Sviluppo di un'applicazione Web per la raccolta e la classificazione, mediante le tecniche dell'Intelligenza Artificiale, di spettri di massa batterici ottenuti con lo spettrometro Maldi-Tof. Tesi di Laurea

Chambers, L., Yang, Y., Littier, H., Ray, P., Zhang, T., Pruden, A., et al. (2015) Metagenomic analysis of antibiotic resistance genes in dairy cow feces following therapeutic administration of third generation cephalosporin. PLoS ONE 10:e0133764. doi: 10.1371/journal.pone.0133764. PMID: 26258869; PMCID: PMC4530880

Chen F, Cheng G, Xu Y, Wang Y, Xia Q, Hu S. (2020) Rumen Microbiota Distribution Analyzed by High-Throughput Sequencing After Oral Doxycycline Administration in Beef Cattle. Front Vet Sci. 2020 Jun 2;7:251. doi: 10.3389/fvets.2020.00251. PMID: 32582771; PMCID: PMC7280444

Cheng W. N., Han S. G. (2020) Bovine mastitis: risk factors, therapeutic strategies, and alternative treatments — A review. Asian-Australasian Journal of Animal Sciences (AJAS); 33(11): 1699-1713. doi: 10.5713/ajas.20.0156. Epub 2020 May 12. PMID: 32777908; PMCID: PMC7649072

Church D.C. (1998) The Ruminant Animal: Digestive Physiology and Nutrition

Cobo-Ángel C, Jaramillo-Jaramillo AS, Lasso-Rojas LM, Aguilar-Marin SB, Sanchez J, Rodriguez-Lecompte JC, Ceballos-Márquez A, Zadoks RN. (2018) Streptococcus agalactiae is not always an obligate intramammary pathogen: Molecular epidemiology of GBS from milk, feces and environment in Colombian dairy herds. PLoS One. 2018 Dec 10;13(12):e0208990. doi: 10.1371/journal.pone.0208990. PMID: 30532177; PMCID: PMC6287850

Colla S., Pranovi F. (2014) Analisi comparativa di indicatori applicati alla comunità macrobentonica della laguna di Venezia, Biol. Mar. Mediterr., 21, 150-153

Cray C., Zaias J. (2009) Altman N.H. Acute phase response in animals: a review. Comp Med. 2009 Dec;59(6):517-26. PMID: 20034426; PMCID: PMC2798837

Cross R., Ling C, Day NP., McGready R., Paris DH. (2016) Revisiting doxycycline in pregnancy and early childhood–time to rebuild its reputation? Expert Opin Drug Saf. 15:367–82. doi: 10.1517/14740338.2016.1133584. Epub 2016 Jan 25. PMID: 26680308; PMCID: PMC4898140

Croxatto A., Prod'hom G., Greub G. (2011) Applications of MALDI-TOF mass spectrometry in clinical diagnostic microbiology. FEMS Microbiol Rev 36 (2012) 380–407. doi: 10.1111/j.1574-6976.2011.00298.x. Epub 2011 Aug 22. PMID: 22092265

Cryan J. F., Dinan T. G., (2012) Mind-altering microorganisms: the impact of the gut microbiota on brain and behavior. Nature Reviews Neuroscience, 13, 701-712. doi: 10.1038/nrn3346. Epub 2012 Sep 12. PMID: 22968153

Cubeddu T., Cacciotto C., Pisanu S., Tedde V., Alberti A., Pittau M., Dore S., Cannas A., Uzzau S., Rocca S., Addis M.F. (2017) Cathelicidin production and release by mammary epithelial cells during infectious mastitis. Veterinary Immunology and Immunopathology Volume 189, July 2017, Pages 66-70 doi: 10.1016/j.vetimm.2017.06.002. Epub 2017 Jun 17. PMID: 28669389.

Daghrir R, Drogui P. (2013) Tetracycline antibiotics in the environment: a review. *Environ Chem Lett.* 11:209–27. doi: 10.1007/s10311-013-0404-8

Dallago C., Cardazzo B., Pindo M., De Filippo C. (2015) Analisi metagenomica del microbiota intestinale di primati (red Colobus). Tesi di Laurea, Università degli Studi di Padova

D'Costa V.M., McGrann K.M., Hughes D.W. and Wright G.D. (2006). Sampling the antibiotic resistome. Science, 311, 374–377. doi: 10.1126/science.1120800. PMID: 16424339

De Olives A.M., C. Peris, M.P. Molina. (2020) Effect of subclinical mastitis on the yield and cheese-making properties of ewe's milk. Small Ruminant Research. doi.org: 10.1016/j.smallrumres.2019.106044

De Visscher A., Supré K., Haesebrouck F., Zadoks R.N., Piessens V., Van Coillie E., et al. (2014) Further evidence for the existence of environmental and host-associated species of coagulase-negative staphylococci in dairy cattle. Vet Microbiol. 172:466–74. doi: 10.1016/j.vetmic.2014.06.011. Epub 2014 Jun 21. PMID: 25008316

De Vliegher S., Fox L.K., Piepers S., McDougall S., Barkema H.W. (2012) Invited review: Mastitis in dairy heifers: nature of the disease, potential impact, prevention, and control. Journal of Dairy Science", vol. 95, 2012, pp. 1025–40. doi: 10.3168/jds.2010-4074. PMID: 22365187

Derakhshani H., Fehr K.B., Sepehri S., Francoz D., De Buck J., Barkema H.W., Plaizier J.C., Khafipour E. (2018) Invited review: Microbiota of the bovine udder: Contributing factors and potential implications for udder health and mastitis susceptibility. Journal of Dairy Science Volume 101, Pages: 10605- 10625. doi: 10.3168/jds.2018-14860. Epub 2018 Oct 3. PMID: 30292553

Dohoo IR, Smith J, Andersen S, Kelton DF, Godden S. (2011) Mastitis Research Workers' Conference. Diagnosing intramammary infections: evaluation of definitions based on a single

milk sample. J Dairy Sci. 2011 Jan;94(1):250-61. doi: 10.3168/jds.2010-3559. PMID: 21183035

Duse A., Waller K.P., Emanuelson U., Unnerstad H.E., Persson Y. and Bengtsson B. (2015) Risk factors for antimicrobial resistance in fecal Escherichia coli from preweaned dairy calves. Journal of Dairy Science, 98, 500–516. doi: 10.3168/jds.2014-8432. Epub 2014 Nov 14. PMID: 25465547

Fakruddin, M., Mannan K. (2013) Methods for analyzing diversity of microbial communities in natural environments. Ceylon. J. Sci. 2013, 42, 19–33

Fan P., Kim M., Liu G., Zhai Y., Liu T., Driver J.D., Jeong K.C. (2021) The Gut Microbiota of Newborn Calves and Influence of Potential Probiotics on Reducing Diarrheic Disease by Inhibition of Pathogen Colonization. Front Microbiol. 2021 Oct 21;12:772863. doi: 10.3389/fmicb.2021.772863. PMID: 34745079; PMCID: PMC8567051

FIL-IDF (International Dairy Federation), (2011) Suggested Interpretation of Mastitis Terminology (revision on Bulletin of IDF N.° 338/1999) in Bulletin of the IDF N° 448/ 2011

Finotello F., Mastrorilli E., Di Camillo B. (2018) Measuring the diversity of the human microbiota with targeted next-generation sequencing. Brief Bioinform. 2018 Jul 20;19(4):679-692. doi: 10.1093/bib/bbw119. PMID: 28025179

Fonty G., Gouet P., Jouany J., Senaud J. (1987) Establishment of the microflora and anaerobic fungi in the rumen of lambs. Journal of General Microbiology. 1988 Mar;34(3):235-41.doi: 10.1139/m88-044. PMID: 3138013

Friman M., Hiitiö H., Niemi M., Holopainen J., Pyörälä S., Simojoki H. (2017) The effect of a cannula milk sampling technique on the microbiological diagnosis of bovine mastitis. Vet J. 226:57–61. doi: 10.1016/j.tvjl.2017.07.003. Epub 2017 Jul 21. PMID: 28911843

Fulghum R.S., Baldwin B.B. and Williams P.P. (1968) Antibiotic susceptibility of anaerobic ruminal bacteria. Applied Microbiology, 1968 Feb;16(2):301-7. doi: 10.1128/am.16.2.301-307.1968. PMID: 5689514; PMCID: PMC547400

Gioia G., Addis M.F., Goodman L.B., Mitchell P.K., Thompson B., Goodrich E., Moroni P. (2021) Draft genome sequence of Acholeplasma laidlawii isolated from the conjunctiva of a heifer with infectious bovine keratoconjunctivitis. Microbiology Resource Announcements, vol. 10(4), 2021, p. 01345-20. doi: 10.1128/MRA.01345-20. PMID: 33509992; PMCID: PMC7844077

Gomes F., & Henriques M. (2016) Control of bovine mastitis: Old and recent therapeutic approaches. Current Microbiology, 72(4), 377–382. doi: 10.1007/s00284-015-0958-8. Epub 2015 Dec 19. PMID: 26687332

Grönlund U., Hultén C., Eckersall P.D., Hogarth C., Waller K.P. (2003) Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced Staphylococcus aureus mastitis The Journal of Dairy Research; Cambridge Vol. 70, Fasc. 4,(Nov 2003): 379-86. doi: 10.1017/s0022029903006484. PMID: 14649408

Hagnestam-Nielsen C, Emanuelson U, Berglund B, Strandberg E. (2009) Relationship between somatic cell count and milk yield in different stages of lactation. J Dairy Sci. 2009 Jul;92(7):3124-33. doi: 10.3168/jds.2008-1719. PMID: 19528590

Haimerl, P., and Heuwieser, W. (2014) Invited review: antibiotic treatment of metritis in dairy cows: a systematic approach. J. Dairy Sci. 97, 6649–6661. doi: 10.3168/jds.2014-8462. Epub 2014 Sep 11. PMID: 25218751

Hashim, H. O., and Al-Shuhaib, M. B. S. (2019) Exploring the potential and limitations of PCR-RFLP and PCR-SSCP for SNP detection: A review. J. Appl. Biotechnol. Rep. 6, 137–144. doi: 10.29252/JABR.06.04.02

Heinrichs J. (2005) Rumen development in the dairy calf. Advances in Dairy Technology. Calf and Heifer Rearing, 53-65

Hiitiö H., Riva R., Autio T., Pohjanvirta T., Holopainen J., Pyörälä S., Pelkonen S. (2015) Performance of a real-time PCR assay in routine bovine mastitis diagnostics compared with in-depth conventional culture. J Dairy Res. 2015 May;82(2):200-8. doi: 10.1017/S0022029915000084. Epub 2015 Feb 23. PMID: 25704849

Hiitiö H., Simojoki H., Kalmus P., Holopainen J., Pyörälä S., Taponen S. (2016) The effect of sampling technique on PCR-based bacteriological results of bovine milk samples. J Dairy Sci. 99:6532–6541. doi: 10.3168/jds.2015-10811. Epub 2016 May 18. PMID: 27209134

Huey S., Kavanagh, M., Regan, A. et al. (2021) Engaging with selective dry cow therapy: understanding the barriers and facilitators perceived by Irish farmers. Ir Vet J 74, 28. doi: 10.1186/s13620-021-00207-0. PMID: 34686221; PMCID: PMC8540178

Hysen B., Urs Zaugg, Kurtesh Sherifi, Afrim Hamidi, Muje Gjonbalaj, Skender Muji, and Hajrip Mehmeti (2010). Influence of management and physiological factors on somatic cell count in raw cow milk. Kosova Vet arhiv 80, 173-183

Jernberg, C., Lofmark, S., Edlund, C., and Jansson, J. K. (2010) Long-term impacts of antibiotic exposure on the human intestinal microbiota. *Microbiology* 156, 3216–3223. doi: 10.1099/mic.0.040618-0. Epub 2010 Aug 12. PMID: 20705661

Kanyshkova T. G., Buneva, V. N., & Nevinsky, G. A. (2001) Lactoferrin and its biological functions. Biochemistry (Moscow), 66(1), 1–7.doi:10.1023/a:1002817226110. PMID: 11240386

Keane O.M., Budd K.E., Flynn J. and McCoy F. (2013) Increased detection of mastitis pathogens by real-time PCR compared to bacterial culture. Veterinary Record 2013;173:268 doi: 10.1136/vr.101598. Epub 2013 Aug 23. PMID: 23976784

Kim Y., Choi S.J., Choi C. (2017) An Efficient PCR-RFLP Method for the Rapid Identification of Korean Pyropia Species. Molecules. Dec 8;22(12):2182. doi: 10.3390/molecules22122182. PMID: 29292786; PMCID: PMC6149700

Konopka A. (2009) What is microbial community ecology? ISME J. 2009 Nov;3(11):1223-30. doi: 10.1038/ismej.2009.88. Epub 2009 Aug 6. PMID: 19657372

Korpela, K., Salonen, A., Virta, L. J., Kekkonen, R. A., Forslund, K., Bork, P., et al. (2016) Intestinal microbiome is related to lifetime antibiotic use in Finnish pre-school children. *Nat. Commun.* 7:10410. doi: 10.1038/ncomms10410. PMID: 26811868; PMCID: PMC4737757

Koskinen M.T., Wellenberg G.J., Sampimon O.C., Holopainen J., Rothkamp A., Salmikivi L., van Haeringen W.A., Lam T.J., Pyörälä S. (2010) Field comparison of real-time polymerase chain reaction and bacterial culture for identification of bovine mastitis bacteria. J Dairy Sci. 2010 Dec;93(12):5707-15. doi: 10.3168/jds.2010-3167. PMID: 21094742

Krömker V., Leimbach S. (2017) Mastitis treatment - Reduction in antibiotic usage in dairy cows. Reprod Domest Anim. 2017 Aug;52 Suppl 3:21-29. doi: 10.1111/rda.13032. PMID: 28815847

Krömker V., Reinecke F., Paduch J.H., Grabowski N. (2014) Bovine Streptococcus uberis intramammary infections and mastitis. Clinical Microbiology, vol. 3(4), 2014, p. 157

Kuipers A., Koops W.J., Wemmenhove H. (2016) Antibiotic use in dairy herds in the Netherlands from 2005 to 2012. Journal of Dairy Science, Vol. 99 (2): 1632-1648. doi: 10.3168/jds.2014-8428. Epub 2015 Dec 17. PMID: 26709178

Lai IH, Tsao JH, Lu YP, Lee JW, Zhao X, Chien FL, Mao SJ. (2009) Neutrophils as one of the major haptoglobin sources in mastitis affected milk. Vet Res. 2009 May-Jun;40(3):17. doi: 10.1051/vetres:2008055. Epub 2008 Dec 19. PMID: 19094922; PMCID: PMC2695032

Leroy F., Van Coillie E., Braem G., Piessens V., Verbist B., De Vuyst L., et al. (2015) Short communication: subtyping of Staphylococcus haemolyticus isolates from milk and corresponding teat apices to verify the potential teat-skin origin of intramammary infections in dairy cows. J Dairy Sci. 98:7893–8. doi: 10.3168/jds.2015-9415. Epub 2015 Aug 20. PMID: 26298760

Little A. E. F., Robinson C. J., Peterson S. B., Raffa K. F., Handelsman J. (2008) Rules of engagement: interspecies interactions that regulate microbial communities. Annual Review of Microbiology, 62, 375-401. doi: 10.1146/annurev.micro.030608.101423. PMID: 18544040

Liu, J., Zhao, Z., Orfe, L., Subbiah, M., and Call, D. R. (2016) Soil-borne reservoirs of antibiotic-resistant bacteria are established following therapeutic treatment of dairy calves. *Environ. Microbiol.* 18, 557–564. doi: 10.1111/1462-2920.13097. Epub 2015 Dec 21. PMID: 26486254

Lopez-Benavides M.G., Dohoo I., Scholl D., Middleton J.R., Perez R. (2012) Interpreting bacteriological culture results to diagnose bovine intramammary infections. National Mastitis Council Research Committee. Report

Ma Y, Khan MZ, Xiao J, Alugongo GM, Chen X, Li S, Wang Y, Cao Z. (2022) An Overview of Waste Milk Feeding Effect on Growth Performance, Metabolism, Antioxidant Status and Immunity of Dairy Calves. Front Vet Sci. 2022 May 17;9:898295. doi: 10.3389/fvets.2022.898295. PMID: 35656173; PMCID: PMC9152456

Malmuthuge N., Chen Y., Laksiri Y. C., Le Luo G. (2015) Heat-treated colostrum feeding promotes beneficial bacteria colonization in the small intestine of neonatal calves. Journal of Dairy Science, 98, 8044–8053. doi: 10.3168/jds.2015-9607. Epub 2015 Sep 3. PMID: 26342981

Malmuthuge N., Nilusha, Le Luo G. (2017) Understanding the Gut Microbiome of Dairy Calves: Opportunities to Improve Early-Life Gut Health, Journal of Dairy Science, 100, 5996-6005. doi: 10.3168/jds.2016-12239. Epub 2017 May 10. PMID: 28501408

Manichanh, C., Reeder, J., Gibert, P., Varela, E., Llopis, M., Antolin, M., et al. (2010) Reshaping the gut microbiome with bacterial transplantation and antibiotic intake. Genome Res. 20, 1411–1419. doi: 10.1101/gr.107987.110. Epub 2010 Aug 24. PMID: 20736229; PMCID: PMC2945190

Marchesi J., Ravel J. (2015) The vocabulary of microbiome research: a proposal. Microbiome, 3, 31. doi: 10.1186/s40168-015-0094-5. PMID: 26229597; PMCID: PMC4520061

Martin M. (2011) Cutadapt removes adapter sequences from high-throughput sequencing reads. Embnet J. 17:10–12. doi: 10.14806/ej.17.1.200

Martinez J.L., Coque T.M. and Baquero F. (2015) What is a resistance gene? Ranking risk in resistomes. Nature Reviews: Microbiology, 13, 116–123. doi: 10.1038/nrmicro3399. Epub 2014 Dec 15. PMID: 25534811

Masson P.L., Heremans J.F., Schonne E. (1969) Lactoferrin, an iron-binding protein in neutrophilic leukocytes. The Journal of Experimental Medicine, 130, 643–658. doi: 10.1084/jem.130.3.643. PMID: 4979954; PMCID: PMC2138704

McDougall S., Parker K. I., Heuer C., & Compton C. W. R. (2009) A review of prevention and control of heifer mastitis via non-antibiotic strategies. Veterinary Microbiology, 134(1), 177–185. doi: 10.1016/j.vetmic.2008.09.026. Epub 2008 Sep 12. PMID: 18986782

Medrano-Galarza C., Gibbons J., Wagner S., de Passillé A.M., Rushen J. (2012) Behavioral changes in dairy cows with mastitis. J Dairy Sci. 2012 Dec;95(12):6994-7002. doi: 10.3168/jds.2011-5247. Epub 2012 Oct 3. PMID: 23040015

Minato H., Otsuka M., Shirasaka S., Itabashi H., Mitsumori M. (1992). Colonization of microorganism in the rumen of young calves. The Journal of General and Applied Microbiology, 38, 447-456. DOI: 10.2323/jgam.38.447

Misra N., Wines T.F., Knopp C.L., Hermann R., Bond L., Mitchell B., McGuire M. A., Tinker J. K. (2018). Immunogenicity of a Staphylococcus aureus-cholera toxin A2/B vaccine for bovine mastitis. Vaccine 36 (2018) 3513–3521. doi: 10.1016/j.vaccine.2018.04.067. Epub 2018 May 5. PMID: 29739718; PMCID: PMC6014625

Mørk T., Jørgensen H.J., Sunde M., Kvitle B., Sviland S., Waage S., et al. (2012) Persistence of staphylococcal species and genotypes in the bovine udder. Vet Microbiol. 159:171–80. doi: 10.1016/j.vetmic.2012.03.034. Epub 2012 Mar 30. PMID: 22503603

Moroni P., Welcome F., Addis M.F., (2016). Etiology, diagnosis and control of mastitis in dairy herds in Achieving sustainable production of milk Volume 3 a cura di John Webster, ed. Burleigh Dodds Science Publishing, London, UK

Morrow J.F., Stearman R.S., Peltzman C.G., Potter D. (1981). Induction of hepatic synthesis of serum amyloid a protein and actin vol.78, No.8,pp.4718-4722,August1981 Biochemistry. doi: 10.1073/pnas.78.8.4718. PMID: 6946420; PMCID: PMC320234

Morvan B., Dore J., Foucat L., Fonty G., Gouet P. (1994) Establishment of hydrogen-utilizing bacteria in the rumen of the newborn lamb. 1994, Microbiology Letters, 117, 3, 249-256. doi: 10.1016/0378-1097(94)90567-3. PMID: 8200502

Nagaraja T.G. and Taylor M.B. (1987). Susceptibility and resistance of ruminal bacteria to antimicrobial feed additives. Applied and Environmental Microbiology, 53, 1620–1625. doi: 10.1128/aem.53.7.1620-1625.1987. PMID: 3116929; PMCID: PMC203920

Nobel, Y. R., Cox, L. M., Kirigin, F. F., Bokulich, N. A., Yamanishi, S., Teitler, I., et al. (2015) Metabolic and metagenomic outcomes from early-life pulsed antibiotic treatment. *Nat. Commun.* 6:7486. doi: 10.1038/ncomms8486. doi: 10.1038/ncomms8486. PMID: 26123276; PMCID: PMC4491183

Nobrega D.B., Naushad S., Naqvi S.A., Condas LAZ, Jean P., Sanders J. (2018) Prevalence and genetic basis of antimicrobial resistance in non- aureus staphylococci isolated from Canadian Dairy Herds. Front Microbiol. 9:256. doi: 10.3389/fmicb.2018.00256. PMID: 29503642; PMCID: PMC5820348

Nonnemann B., Lyhs U., Svennesen L., Kristensen K.A., Klaas I.C., Pedersen K. (2019) Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. J Dairy Sci 102:2515–2524. doi: 10.3168/jds.2018-15424. Epub 2019 Jan 11. PMID: 30639010

O'Reilly E.L., Eckersall P.D. (2014) Acute phase proteins: a review of their function, behaviour and measurement in chickens. World's Poultry Science Journal, 70:1, 27-44, doi: 10.1017/S0043933914000038

O'Neill, J. (2014) Antimicrobial resistance: Tackling a crisis for the health and wealth of nations. Review on Antimicrobial Resistance, 2014, 1–16, http://amr-review.org/Publications

Oviaño M., Rodríguez-Sánchez B. (2021) MALDI-TOF mass spectrometry in the 21st century clinical microbiology laboratory. Enferm Infecc Microbiol Clin (Engl Ed). 2021 Apr;39(4):192-200. English, Spanish. doi: 10.1016/j.eimc.2020.02.027. Epub 2020 Apr 25. PMID: 32345492

Panneerchelvam S., Norazmi M.N. (2003) Forensic DNA profiling and database. Malays J Med Sci. 2003 Jul;10(2):20-6. PMID: 23386793; PMCID: PMC3561883.

Pereira R.V., Siler J.D., Bicalho R.C. and Warnick L.D. (2014) In vivo selection of resistant E. coli after ingestion of milk with added drug residues. PLoS ONE, 9, e115223. doi: 10.1371/journal.pone.0115223. PMID: 25506918; PMCID: PMC4266680

Pesce A., Garofalo F., Coppa P., De Marco G., Cioffi B., Salzano C., Guarino A. (2013) Large Animal Review 2013; 19: 162-164 https://vetjournal.it/images/archive/pdf_riviste/4617.pdf

Peton V., Le Loir Y. (2014) Staphylococcus aureus in veterinary medicine. Infection, Genetics and Evolution, vol. 21, 2014, pp. 602–615. doi: 10.1016/j.meegid.2013.08.011. Epub 2013 Aug 23. PMID: 23974078

Petrovski K.R., Trajcev M., Buneski G. (2006) A review of the factors affecting the costs of bovine mastitis. J S Afr Vet Assoc. 2006 Jun;77(2):52-60. doi: 10.4102/jsava.v77i2.344. PMID: 17120619

Piccart K., Verbeke J., De Visscher A., Piepers S., Haesebrouck F., De Vliegher S. (2016) Local host response following an intramammary challenge with Staphylococcus fleurettii and different strains of Staphylococcus chromogenes in dairy heifers. Vet Res. 47:1–11. doi: 10.1186/s13567-016-0338-9. PMID: 27176792; PMCID: PMC4866078

Piepers S., De Meulemeester L., de Kruif A., Opsomer G., Barkema H.W., De Vliegher S. (2007) Prevalence and distribution of mastitis pathogens in subclinically infected dairy cows in Flanders, Belgium. J Dairy Res. 74:478–83. doi: 10.1017/S0022029907002841. Epub 2007 Oct 12. PMID: 17931457

Pisanu S., Cubeddu T, Pagnozzi D, Rocca S, Cacciotto C, Alberti A, Marogna G, Uzzau S, Addis MF. (2015) Neutrophil extracellular traps in sheep mastitis. Vet Res. 2015 Jun 18;46(1):59. doi: 10.1186/s13567-015-0196-x. PMID: 26088507; PMCID: PMC4471908

Pol M., Ruegg P. (2007) Relationship Between Antimicrobial Drug Usage and Antimicrobial Susceptibility of Gram-Positive Mastitis Pathogens. J. Dairy Sci. 2007;90:262–273. doi: 10.3168/jds.S0022-0302(07)72627-9. PMID: 17183094

Pol M., and P.L. Ruegg. (2007) Treatment practices and quantification of antimicrobial drug usage in conventional and organic dairy farms in Wisconsin. J Dairy Sci. 90: 249–261. doi: 10.3168/jds.S0022-0302(07)72627-9. PMID: 17183094

Pulina G., Milán M. J., Lavín M. P., Theodoridis A., Morin E., Capote J., Caja, G. (2018) Invited review: Current production trends, farm structures, and economics of the dairy sheep and goat sectors. Journal of Dairy Science, 101(8), 6715–6729. doi: 10.3168/jds.2017-14015. Epub 2018 May 30. PMID: 29859690

Radostits O.M., Gay C.C., Blood D.C., Hinchcliff K.W. (2000) Veterinary medicine, a textbook of the disease of cattle, sheep, pigs, goats and horses. W.B. Saunders Company Ltd., London, United Kingdom 603-700

Rasheed A., Usman T., Niaz K. (2020) A review on bovine mastitis with special focus on CD4 as a potential candidate gene for mastitis resistance – a review. Ann. Anim. Sci., Vol. 20, No. 3 (2020) 735– 755. doi: 10.2478/aoas-2020-00

Rasmussen H.B. (2011) Restriction fragment length polymorphism analysis of PCR-amplified fragments (PCR-RFLP) and gel electrophoresis- valuable tool for genotyping and genetic fingerprinting. InTech; 2011. doi:10.5772/37724

Reczyńska D., Zalewska M., Czopowicz M. et al. (2018) Small ruminant lentivirus infection influences expression of acute phase proteins and cathelicidin genes in milk somatic cells and peripheral blood leukocytes of dairy goats. Vet Res 49, 113. doi: 10.1186/s13567-018-0607-x. PMID: 30424807; PMCID: PMC6234539

Reyher K.K., Dohoo I.R. (2011). Diagnosing intramammary infections: evaluation of composite milk samples to detect intramammary infections. J Dairy Sci. 2011 Jul;94(7):3387-96. doi: 10.3168/jds.2010-3907. PMID: 21700024

Ricchi M., De Cicco C., Buzzini P., Cammi G., Arrigoni N., Cammi M., Garbarino C. (2013) First outbreak of bovine mastitis caused by Prototheca blaschkeae. Veterinary Microbiology, vol. 162(2-4), 2013, pp. 997-999. doi: 10.1016/j.vetmic.2012.11.003. Epub 2012 Nov 9. PMID: 23201242

Ricci A., Allende A., Bolton D., Chemaly M., Davies R. et al. (2017) Risk for the Development of Antimicrobial Resistance (AMR) Due to Feeding of Calves with Milk Containing Residues of Antibiotics. EFSA J. 2017 Jan 27;15(1):e04665. doi: 10.2903/j.efsa.2017.4665. PMID: 32704309; PMCID: PMC7372110

Rollin, E., Dhuyvetter, K. C., & Overton, M. W. (2015) The cost of clinical mastitis in the first 30 days of lactation: An economic modeling tool. Preventive Veterinary Medicine, 122(3), 257–264. doi: 10.1016/j.prevetmed.2015.11.006. Epub 2015 Nov 7. PMID: 26596651

Ruegg P.L. (2011) Managing mastitis and producing quality milk. in Dairy production medicine. Risco C.A. e Melendez P.R., ed. John Wiley & Sons, West Sussex, UK, 2011, pp. 207-232

Ruegg P.L., Pantoja J.C.F. (2013) Understanding and using somatic cell counts to improve milk quality. Irish Journal of Agricultural and Food Research, vol. 52, 2013, pp. 101–117

Ruegg P.L. (2003) Practical food safety interventions for dairy production. Journal of Dairy Science, vol. 86(E. Suppl.), pp. E1–E9

Sadek K., Saleh E. and Ayoub M. (2016) Selective, reliable blood and milk biomarkers for diagnosing clinical and subclinical bovine mastitis. Tropical Animal Health and Production 49, 431–437. doi: 10.1007/s11250-016-1190-7. Epub 2016 Dec 3. PMID: 27915437

Sampimon O.C., Barkema H.W., Berends IMGA, Sol J, Lam TJGM (2009) Prevalence and herd-level risk factors for intramammary infection with coagulase-negative staphylococci in Dutch dairy herds. Vet Microbiol. 134:37–44. doi: 10.1016/j.vetmic.2008.09.010. Epub 2008 Sep 11. PMID: 18977613

Sarangi A. N., A. Goel, and R. Aggarwal (2019) Methods for studying gut microbiota: a primer for physicians. Journal of Clinical and Experimental Hepatology, vol. 9, no. 1, pp. 62–73, 2019. doi: 10.1016/j.jceh.2018.04.016. Epub 2018 May 4. PMID: 30774267; PMCID: PMC6363981

Saunders W.B. (2017) 20 - Diseases of the Mammary Gland. Pages 1904-2001. Veterinary Medicine (Eleventh Edition). Editor(s): Peter D. Constable, Kenneth W. Hinchcliff, Stanley H. Done, Walter Grünberg. ISBN 9780702052460

Schmitz S., Pfaffl M.W., Meyer H.H.D. Bruckmaier R.M. (2004) Short-term changes of mRNA expression of various inflammatory factors and milk proteins in mammary tissue during LPS-induced mastitis. Domest. Anim. Endocrinol. 2004; 26: 111-126. doi: 10.1016/j.domaniend.2003.09.003. PMID: 14757184

Schroeder J.W. (2012) Bovine mastitis and milking management. Extension Dairy Specialist, vol. AS1129 (Revised), pp. 1-16

Schukken YH, Hertl J, Bar D, Bennett GJ, González RN, Rauch BJ, Santisteban C, Schulte HF, Tauer L, Welcome FL, Gröhn YT. (2009) Effects of repeated Gram-positive and Gram-negative clinical mastitis episodes on milk yield loss in Holstein dairy cows. J Dairy Sci. 2009 Jul;92(7):3091-105. doi:10.3168/jds.2008-1557. PMID: 19528587

Schukken Y.H., Günther J., Fitzpatrick J., Fontaine M.C., Goetze L., Holst O., Leigh J., Petzl W., Schuberth H.J., Sipka A., Smith D.G., Quesnell R., Watts J., Yancey R., Zerbe H., Gurjar A., Zadoks R.N., Seyfert H.M., members of the Pfizer mastitis research consortium. (2011) Host-response patterns of intramammary infections in dairy cows in Veterinary Immunology and Immunopathology, vol. 144(3–4), pp. 270–289. doi: 10.1016/j.vetimm.2011.08.022. Epub 2011 Sep 10. PMID: 21955443

Schukken Y.H., Schmitt-Van De Leemput E., Moroni P., Welcome F., Gurjar A., Zurakowski M., Gutierrez C. (2012) Contagious or Environmental - A Herd Diagnosis. In Proceedings of World Buiatrics Congress, ed. World Association for Buiatrics, Lisbon, Portugal, 2012, pp. 145-148

Schwarz S., Chaslus-Dancla E. (2001) Use of antimicrobials in veterinary medicine and mechanisms of resistance. Vet Res. 32: 201-225. doi: 10.1051/vetres:2001120. PMID: 11432414

Seng P., Drancourt M., Gouriet F., La Scola B., Fournier P.E., Rolain J.M. & Raoult D. (2009) Ongoing revolution in bacteriology: routine identification of bacteria by matrix- assisted laser desorption ionization time-of-flight mass spectrometry. Clin Infect Dis 49: 543–551. doi: 10.1086/600885. PMID: 19583519

Shterzer N., Mizrahi I. (2015) The animal gut as a melting pot for horizontal gene transfer. Can J Microbiol. 2015 Sep;61(9):603-5. doi: 10.1139/cjm-2015-0049. Epub 2015 Apr 20. PMID: 26053634

Shirasawa K., Hirakawa H., Isobe S. (2016) Analytical workflow of double-digest restriction site-associated DNA sequencing based on empirical and in silico optimization in tomato. DNA Res. 2016;23(2):145-153. doi: 10.1093/dnares/dsw004. Epub 2016 Feb 29. PMID: 26932983; PMCID: PMC4833422

Simon L., France Gauvin, Devendra K. Amre, Patrick Saint-Louis, Jacques Lacroix (2004) Serum Procalcitonin and C-Reactive Protein Levels as Markers of Bacterial Infection: A Systematic Review and Meta-analysis. Clinical Infectious Diseases, Volume 39, Issue 2, 15 July 2004, Pages 206–217. doi: 10.1086/421997. Epub 2004 Jul 2. Erratum in: Clin Infect Dis. 2005 May 1;40(9):1386-8. PMID: 15307030

Smolenski G.A., Wieliczko R.J., Pryor S.M., Broadhurst M.K., Wheeler T.T. Haigh B.J. (2011) The abundance of milk cathelicidin proteins during bovine mastitis. Vet Immunopathol;

143(21774993): 125-130.doi: 10.1016/j.vetimm.2011.06.034. Epub 2011 Jul 2. PMID: 21774993

Surette M. and Wright G.D. (2017) Lessons from the environmental antibiotic resistome. Annual Reviews of Microbiology, 71:309-329. doi: 10.1146/annurev-micro-090816-093420. Epub 2017 Jun 28. PMID: 28657887

Taghdiri M., Karim G., Safi S., Rahimi Foroushani A., Motalebi A. (2018) Study on the accuracy of milk amyloid A test and other diagnostic methods for identification of milk quality. Vet Res Forum. 2018;9(2):179-185. doi: 10.30466/VRF.2018.30830. Epub 2018 Jun 15. PMID: 30065807; PMCID: PMC6047578

Taponen S., Björkroth J., Pyörälä S. (2008) Coagulase-negative staphylococci isolated from bovine extramammary sites and intramammary infections in a single dairy herd. J Dairy Res. 75:422–9. doi: 10.1017/S0022029908003312. Epub 2008 Aug 14. PMID: 18700996

Taponen S, Salmikivi L, Simojoki H, Koskinen MT, Pyörälä S. (2009) Real-time polymerase chain reaction-based identification of bacteria in milk samples from bovine clinical mastitis with no growth in conventional culturing. J Dairy Sci. 2009 Jun;92(6):2610-7. doi: 10.3168/jds.2008-1729. PMID: 19447993

Thames C.H., Pruden A., James R.E., Ray P.P. and Knowlton K.F. (2012) Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. Frontiers in Microbiology, 3, 139. doi: 10.3389/fmicb.2012.00139. PMID: 22514550; PMCID: PMC3322659

Ursell L. K., Metcalf J. L., Wegener Parfrey L., Knight R. (2012) Defining the Human Microbiome, Nutrition Reviews, 70, 38-4. doi: 10.1111/j.1753-4887.2012.00493.x. PMID: 22861806; PMCID: PMC3426293

Vanderhaeghen W., Piepers S., Leroy F., Van Coillie E., Haesebrouck F., De Vliegher S. (2014) Invited review: effect, persistence, and virulence of coagulase-negative Staphylococcus species associated with ruminant udder health. J Dairy Sci. 97:5275–93. doi: 10.3168/jds.2013-7775. Epub 2014 Jun 18. PMID: 24952781

Vasquez, A. K., Nydam, D. V., Capel, M. B., Ceglowski, B., Rauch, B. J., Thomas, M. J., et al. (2016) Randomized noninferiority trial comparing 2 commercial intramammary antibiotics for the treatment of nonsevere clinical mastitis in dairy cows. J. Dairy Sci. 99, 8267–8281. doi: 10.3168/jds.2016-11258. Epub 2016 Aug 10. PMID: 27522408

Viguier C., Arora S., Gilmartin N., Welbeck K., O'Kennedy R. (2009) Mastitis detection: current trends and future perspectives. Trends Biotechnol. 2009 Aug;27(8):486-93. doi: 10.1016/j.tibtech.2009.05.004. Epub 2009 Jul 16. PMID: 19616330

Vlek A.L., Bonten M.J., and Boel C.H. (2012) Direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry improves appropriateness of antibiotic treatment of bacteremia. PLoS One7:e32589. doi: 10.1371/journal.pone.0032589. Epub 2012 Mar 16. PMID: 22438880; PMCID: PMC3306318

Walters KE, Martiny JBH. (2020) Alpha-, beta-, and gamma-diversity of bacteria varies across habitats. PLoS One. 2020 Sep 23;15(9):e0233872. doi: 10.1371/journal.pone.0233872. PMID: 32966309; PMCID: PMC7510982

Weber, A., Weber, A. T., McDonald, T. L., & Larson, M. A. (2006) Staphylococcus aureus lipotechoic acid induces differential expression of bovine serum amyloid A3(SAA3) by mammary epithelial cells: Implications for early diagnosis of mastitis. Veterinary Immunology and Immunopathology, 109(1-2), 79– 83. doi: 10.1016/j.vetimm.2005.07.023. Epub 2005 Aug 31. PMID: 16139367

Whyte D., Walmsley M., Liew A., Claycomb R., Mein G. (2005) Chemical and rheological aspects of gel formation in the California Mastitis Test. Journal of Dairy Research, vol. 72, pp. 115–121. doi: 10.1017/s0022029904000561. PMID: 15747739

Wichmann, F., Udikovic-Kolic, N., Andrew, S., and Handelsman, J. (2014) Diverse antibiotic resistance genes in dairy cow manure. *mBio* 5:e01017–13. doi: 10.1128/mBio.01017-13. PMID: 24757214; PMCID: PMC3993861

Woese C., Fox G. (1977) Phylogenetic structure of the prokaryotic domain: The primary kingdoms. Proceedings of the National Academy of Sciences 74(11): 5088–5090. doi: 10.1073/pnas.74.11.5088. PMID: 270744; PMCID: PMC432104

Woolhouse M.E, and Ward M.J., (2013) Sources of antimicrobial resistance**.** Science 341, 1460–1461. doi: 10.1126/science.1243444. Epub 2013 Sep 12. PMID: 24030495

Yan, S. S., and J. Gilbert. (2004) Antimicrobial drug delivery in food animals and microbial food safety concerns: An overview of in vitro and in vivo factors potentially affecting the animal gut microflora. Adv. Drug Deliv. Rev. 56:1497–1521. https://doi.org/101016/j.addr.2004.02.010. PMID: 15191795

Yanez-Ruiz D., Abecia L., Newbold C., (2015) Manipulating rumen microbiome and fermentation through interventions during early life: a review. Frontiers in Microbiology, 6: 1133. 2015. doi: 10.3389/fmicb.2015.01133. PMID: 26528276; PMCID: PMC4604304

Young-Speirs, M., Drouin, D., Cavalcante, P. A., Barkema, H. W., & Cobo, E. R. (2018). Host defense cathelicidins in cattle: types, production, bioactive functions, and potential therapeutic and diagnostic applications. International Journal of Antimicrobial Agents, 51(6), 813-821. doi: 10.1016/j.ijantimicag.2018.02.006. Epub 2018 Feb 21. PMID: 29476808

Yu G-M, Kubota H., Okita M., Maeda T. (2017) The anti-inflammatory and antioxidant effects of melatonin on LPS-stimulated bovine mammary epithelial cells. PLoS ONE 12(5): e0178525. doi: 10.1371/journal.pone.0178525. PMID: 28542575; PMCID: PMC5444821

Zhang, GW, L, SJ, YoshimuraY, IsobeN. (2014) Expression of cathelicidins mRNA in the goat mammary gland and effect of the intramammary infusion of lipopolysaccharide on milk cathelicidin-2 concentration Veterinary Microbiology Volume 170, Issues 1–2, 14 May 2014, Pages 125-134. doi: 10.1016/j.vetmic.2014.01.029. Epub 2014 Feb 7. PMID: 24572177

Zheng Z., Hou Y., Cai Y., Zhang Y., Li Y., Zhou M. (2015) Whole-genome sequencing reveals that mutations in myosin-5 confer resistance to the fungicide phenamacril in Fusarium graminearum. Sci Rep. 2015 Feb 4;5:8248. doi: 10.1038/srep08248. PMID: 25648042; PMCID: PMC5389027

Zou Y, Wang Y, Deng Y, Cao Z, Li S, Wang J. (2017) Effects of feeding untreated, pasteurized and acidified waste milk and bunk tank milk on the performance, serum metabolic profiles, immunity, and intestinal development in Holstein calves. J Anim Sci Biotechnol. 8:53. 10.1186/s40104-017-0182-4

Aims

Mastitis is one of the most common and costly diseases affecting dairy ruminantsand its sensitive and specific detection is of significant importance. Monitoring and maintaining udder health is critical for cows' and small ruminants' welfare and dairy production yield and quality. The clinical evaluation of udder and mammary secretions is typically combined with the milk SCC and often accompanied by its bacteriological culture to identify the causative microorganism. Several non-enzymatic milk proteins, including M-SAA, HP, CATH, and LF, have been studied as alternative biomarkers of mastitis for their relationship with mammary gland inflammation and immunoassay techniques have been developed for detection with varying degrees of success.

Concerning the causative agents, infectious mastitis outbreaks can be caused by various bacterial species. Among these, NAS and streptococci are common causes of IMI. According to subclinical bovine mastitis studies, some species of NAS are more associated with IMI, subclinical mastitis, and increased SCC than others. Obtaining accurate species information would enable better management choices when these bacteria are isolated from milk.

Concerning the treatment of mastitis, antibiotic therapy continues to play a crucial role. Animals receiving antibiotics produce milk that cannot be marketed. This is considered waste milk (WM), and a convenient option for farmers is using it as feed for young animals. Adding to the risk of selecting resistant bacteria, residual antibiotics might interfere with gut microbiome development and influence gastrointestinal health.

By putting together these issues and challenges, the present work aims to investigate the application of new approaches to improve the knowledge of dairy ruminant mastitis and its related issues in animal management. Specifically, the points addressed are listed below:

I. Applying MALDI-TOF MS and gap PCR-RFLP to identify staphylococci and streptococci species isolated from the milk of sheep and goats with mastitis. We compared the respective results to identify potential problems and solutions.

II. Defining the state-of-the-art non-enzymatic milk markers of mastitis. We conducted a systematic review on milk proteins as mastitis markers for understanding, using a literature search, the implementation of different protein biomarkers in ruminant mastitis diagnosis using immunoassays with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. Our goal was to report systematically and organically what has been published in this specific field in the scientific literature.

III. Assessing the impact of feeding waste milk containing antibiotic residues on the calf gut microbiota. An important issue is the reduction of antibiotic use in animals worldwide and the understanding of their impact on animal health and antimicrobial resistance. We investigated the impact of feeding waste milk with antibiotics obtained from cows with chronic mastitis to dairy calves to evaluate its impact on gut health and microbiota composition to reduce future health issues.

Part I: MALDI-TOF MS and gap PCR–RFLP for species identification of non-*aureus Staphylococcus*, *Mammaliicoccus*, and *Streptococcus* spp. associated with sheep and goat mastitis

During my Ph.D., I had the opportunity to participate in a collaborative research project between the University of Milan and Istituto Zooprofilattico Sperimentale della Sardegna (IZSSA). From January 2021 to May 2022, 204 non-*aureus* staphylococci (NAS) and mammaliicocci (NASM) and 57 streptococci were isolated from sheep and goat milk samples that routinely arrive at the IZSSA laboratories for identification of the IMI agent. Milk samples were cultured following the standard procedures provided by the IZSSA and then identified by MALDI-TOF MS and PCR–RFLP for 261 non-duplicate isolates. A geographic map indicating the collection site of each isolate in the different provinces of Sardinia was created (Figure 15).

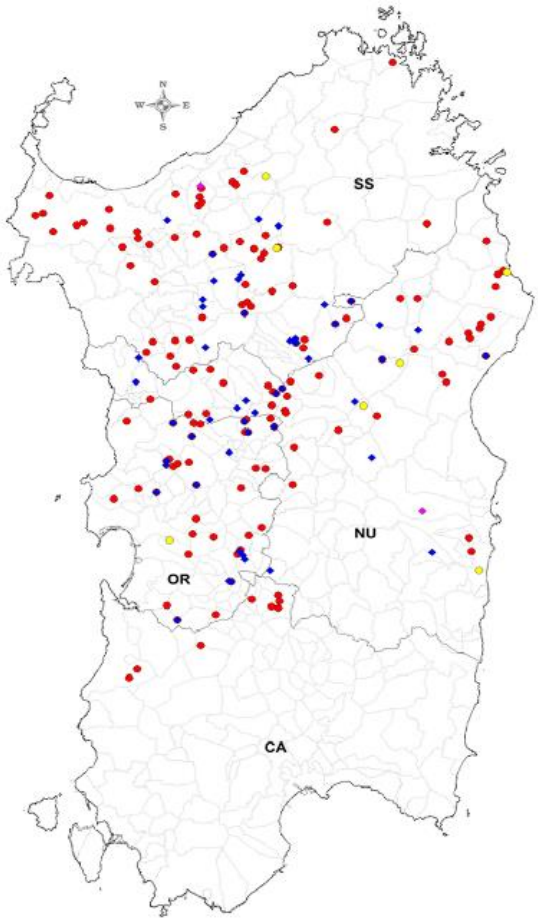

Figure 15: Geographical distribution of all 261 isolates included in this study. The map reports the location of the 204 non-aureus Staphylococcus and Mammaliicoccus (NASM) and 57 Streptococcus isolates in Sardinia, Italy. Each point represents an individual isolate. Circles indicate NASM: red for sheep milk and yellow for goat milk. Triangles indicate streptococci: blue for sheep milk, and fuchsia for goat milk (Reproduced from Rosa et al., 2022)

Our results concerning the species of NASM and streptococci associated with sheep and goat mastitis followed those described as causing small ruminant IMI worldwide. MALDI-TOF MS and *gap* PCR–RFLP provided comparable results for the most prevalent species. *Gap* PCR–RFLP can offer a reliable identification alternative when MALDI-TOF MS is not available, but restriction profiles differing from the validated reference isolates may not be easily resolved by *gap* gene sequencing. Regarding MALDI-TOF MS, implementing the spectrum library with small ruminant strains of *S. haemolyticus* and *S. microti* and their closely related species might improve identification performances. The article has been published as Rosa N.M., Penati M., Fusar-Poli S., Addis M.F., Tola S. Species identification by MALDI-TOF MS and gap PCR-RFLP of non-*aureus* *Staphylococcus*, *Mammaliicoccus*, and *Streptococcus* spp. associated with sheep and goat mastitis. Vet Res. 2022 Oct 15;53(1):84. doi: 10.1186/s13567-022-01102-4. PMID: 36243811; PMCID: PMC9569034.

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Species identification by MALDI-TOF MS and gap PCR–RFLP of non-*aureus* *Staphylococcus*, *Mammaliicoccus*, and *Streptococcus* spp. Associated with sheep and goat mastitis

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Abstract

Staphylococci and streptococci are common causes of intramammary infection in small ruminants, and reliable species identification is crucial for understanding epidemiology and its impact on animal health and welfare. We applied MALDI-TOF MS and *gap* PCR–RFLP to 204 non-aureus staphylococci (NAS), mammaliicocci (NASM), and 57 streptococci isolated from the milk of sheep and goats with mastitis. The top identified NAS was *Staphylococcus epidermidis* (28.9%), followed by *Staph. chromogenes* (27.9%), *haemolyticus* (15.7%), *caprae*, and *simulans* (6.4% each), according to both methods (agreement rate, AR, 100%). By MALDI-TOF MS, 13.2% were *Staph. microti* (2.9%), *xylosus* (2.0%), *equorum, petrasii* and *warneri* (1.5% each), *Staph. sciuri* (now *Mammaliicoccus sciuri*, 1.0%), *arlettae, capitis, cohnii, lentus* (now M. lentus), *pseudintermedius, succinus* (0.5% each), and 3 isolates (1.5%) were not identified. PCR–RFLP showed 100% AR for *Staph. equorum, warneri, arlettae, capitis*, and *pseudintermedius,* 50% for *Staph. xylosus*, and 0% for the remaining NASM. The top identified streptococcus was *Streptococcus uberis* (89.5%), followed by *Strep. dysgalactiae* and *parauberis* (3.5% each) and by *Strep. gallolyticus* (1.8%) according to both methods (AR 100%). MALDI-TOF MS and PCR–RFLP identified only one isolate as a different species. MALDI-TOF MS and PCR–RFLP showed a high level of agreement in

identifying the most prevalent NAS and streptococci causing small ruminant mastitis. G*ap* PCR–RFLP can represent a good identification alternative when MALDI-TOF MS is unavailable. Some issues remain for *Staph. haemolyticus*, minor NAS species including *Staph. microti*, and species of the novel genus *Mammaliicoccus.*

Keywords: Small ruminant, milk, coagulase-negative staphylococci, streptococci, mammaliicocci, NAS, NASM, species identification, *Gap* gene

Introduction

Mastitis is one of the most common and costly diseases affecting dairy sheep and goats., monitoring and maintaining udder health is critical for small ruminant welfare and dairy production yield and quality (Conington et al., 2008; Gelasakis et al., 2015). A wide variety of bacterial species can cause infectious mastitis outbreaks, including the genera *Staphylococcus* (*Staph.*) and *Streptococcus* (*Strep.*) (Gelasakis et al., 2015; Dore et al., 2016). Among *Staphylococcus* spp., non-*aureus* staphylococci (NAS) are the most prevalent and cause mainly subclinical intramammary infection (IMI) in both ewes and goats, leading to significant economic losses and reduced animal welfare (Marogna et al., 2012; Marogna et al., 2010). Recently, based on 16S rRNA sequences, five NAS species were reclassified and assigned to the novel genus *Mammaliicoccus* with *Mammaliicoccus sciuri* as the type species (Madhaiyan et al., 2020). These species are collectively indicated by the acronym NASM. The large NASM bacterial group includes numerous species with different prevalences and epidemiology (Gelasakis et al., 2015; Dore et al., 2016; Bernier Gosselin et al., 2019). From studies on bovine subclinical mastitis, it is clear that some species are more associated with IMI, subclinical mastitis, and somatic cell count (SCC) increase than others, which are instead more related to the farm environment or the mammary gland microbiota (Madhaiyan et al., 2020; De Buck et al., 2021; Oikonomu et al., 2020; Derakhshani et al., 2018). Obtaining precise information on the species of NASM causing IMI and mastitis is crucial for understanding the epidemiology and the respective roles in mammary gland health and disease to make more appropriate management decisions when these bacteria are isolated from the milk of small ruminants (Bernier Gosselin et al., 2019; Vasileiou et al., 2019). In the microbiological laboratory, staphylococci and streptococci isolated from milk samples are mainly identified at the species level using biochemical tests or commercial biochemical galleries. These methods often fail to correctly identify bacterial species of veterinary interest because they have been optimized for the strains associated with human infections (Onni et al., 2010; Onni et al., 2012; Wanecka et al., 2019; Vanderhaeghen et al., 2015). In the last decade, matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF

MS) has emerged as a fast and accurate microbial identification approach (Seng et al., 2010) and is being successfully applied to bacteria isolated from bovine milk (Nonnemann et al., 2019; Hamel et al., 2020; Conesa et al., 2020). There are few reports of its performance in identifying bacteria isolated from small ruminant milk, especially concerning staphylococci and sheep. Identification methods based on molecular rather than phenotypic characteristics can also provide a reliable alternative. The Istituto Zooprofilattico Sperimentale della Sardegna (IZSSA) has developed, comparatively assessed, and implemented a PCR-restriction fragment length polymorphism (PCR–RFLP) method based on PCR amplification of the *gap* gene followed by *Alu*I enzyme digestion for the identification of staphylococci and streptococci isolated from routine diagnosis (Onni et al., 2010; Onni et al., 2012; Rosa et al., 2019). In this study, we applied MALDI-TOF MS and *gap* PCR–RFLP for the species identification of staphylococci and streptococci isolated from the milk of sheep and goats with mastitis. We compared their respective results to identify potential issues and solutions. Bacterial isolates were collected in Sardinia, the region with the largest small ruminant population in Italy.

Materials and methods

Bacterial isolates

From January 2021 to May 2022, 204 NASM and 57 *Streptococcus* spp. were isolated from sheep and goat milk samples that routinely arrived at the IZSSA laboratories for identification of the IMI agent. Milk samples are accompanied by a form with a checkbox indicating the presence of clinical mastitis/visible milk alterations to be selected by the farm veterinarian. Only isolates derived from samples with these characteristics were included in the study. Milk samples were cultured following the standard procedures provided by the IZSSA. 10 μL of milk were seeded in 5% sheep blood agar, incubated at 37 °C, and evaluated at 24 and 48 h. With the growth of more than one morphologically different bacterial colony, identifications were not performed, and the sample was classified as "mixed bacterial flora". Colonies were re-isolated in blood agar and examined with Gram stain, catalase, and coagulase tests to discriminate between staphylococci and streptococci. All isolates were stored at −20°C in Brain Heart Infusion broth (BHI, Beckton-Dickinson, Sparks, MD, USA) containing 20% glycerol until further investigation. Only one NASM or streptococcus isolate was selected from each farm for identification by MALDI-TOF MS and PCR–RFLP for 261 non-duplicate isolates. A geographical map indicating the collection site of each isolate in the different provinces of Sardinia was created with the software Microsoft Power BI using the farm code of each flock. The geo-referenced coordinates were extracted from the Banca Dati Nazionale (BDN) of the Italian Health Ministry.

MALDI-TOF MS for bacterial identification

At the IZSSA, all bacterial isolates were retrieved from the frozen archives, seeded in 5% sheep blood agar, and incubated at 37 °C. All isolates were passaged twice in the solid medium before identification at 24 h of growth. For MALDI-TOF MS analysis, the direct colony transfer protocol was applied. A small amount of an isolated colony was deposited in duplicate wells of disposable target plates using a toothpick, overlaid with 1 µL of α-cyano-4-hydroxycinnamic acid (HCCA) solution in 50% acetonitrile, 47.5% water, 2.5% trifluoroacetic acid (Bruker Daltonik GmbH, Bremen, Germany), and left to dry. The target plates were prepared at the IZZSA in the afternoon, placed in an empty disposable target container once dry, and transported at room temperature to the animal infectious diseases laboratory at the University of Milan (MILab). In the morning of the following day, i.e. within 24 h as recommended by the MALDI Bio-typer (MBT) Bruker user manual, the mass spectra were acquired with the MBT Microflex LT/SH MALDI-TOF mass spectrometer (Bruker Daltonik GmbH) in the positive mode. Each target plate included two spots of Bacterial Test Standard (Bruker Daltonik GmbH). The obtained spectra were interpreted against the MBT Compass Library Revision H (2021), covering 3893 species/entries. The *Staphylococcus* and *Streptococcus* species included in this library revision are listed in Additional file 2. The two *Mammaliicoccus* species *M. sciuri and M. lentus,* were reported as *Staph. sciuri* and *Staph. lentus* in the library revision available at the time of this study. The following similarity log score thresholds were considered (Seng et al., 2010): a log score ≥ 2.0 indicated a reliable species-level identification, while a log score between 1.7 and 2.0 indicated a presumptive species-level identification. Identifications with log scores below 1.7 were considered unreliable. All samples producing scores below 1.7 were processed again with the direct transfer, extended direct transfer, and protein extraction procedures. Specifically, for the extended direct transfer procedure, after depositing a small amount of the isolated colony in duplicate wells of the disposable target plate using a toothpick, the sample was overlaid with 1 µL of 70% formic acid and left to dry before adding the HCCA matrix solution. For the protein extraction, bacteria from 4 isolated colonies were deposited into 300 µL of HPLC-grade water and vortexed. Then, 900 µL of ethanol absolute were added, the tube was vortexed again, and centrifuged for 2 min at maximum speed in a microcentrifuge. The supernatant was removed carefully, and the pellet was left to dry for 10 min at room temperature. Once dried, the pellet was thoroughly resuspended in 25 µL of 70% formic acid, 25 µL of acetonitrile were added, and the suspension was mixed by pipetting. The sample was centrifuged again as above, and 1 µL of supernatant was deposited on the MALDI target and left to dry before overlaying with the HCCA matrix.

DNA extraction and PCR amplification

At the IZSSA, genomic DNA was extracted from all 261 isolates and Type/Reference Strains (T/RS) as described previously (Onni et al. 2010). Species identification was based on PCR amplification of the glyceraldehyde-3-phosphate dehydrogenase gene (*gap*) gene (Rosa et al., 2019; Yugueros et al., 2000). The primers GF-1 (5'- ATGGTTTTGGTAGAATTGGTCGTTTA-3') and GR-2 (5'-GACATTTCGTTATCATACCAAGCTG-3') were used for staphylococci whereas the primers Strept-gap-F (5'-ACTCAAGTGTACGAACAAGT-3') and Strept-gap- R (5'-GTCTTGCATTCCGTCGTAT-3') for streptococci. PCR was performed in a reaction mixture containing 2.5 µL 10×reaction buffer, 1.5 µL dNTPs 1.25 mM, 1 µL of each primer (25 pmol each), 1 µL DNA template, 0.5 µL Fast Taq (Roche, Basel, Switzerland), and distilled water up to 25 µL. Reactions were carried out in an automated DNA thermal cycler (GeneAmp 9700, Applied Biosystems, CA, USA). Amplification conditions: initial denaturation for 5 min at 95 °C followed by 30 cycles of 1 min at 95 °C, 1 min at 50 °C (for staphylococci)/1 min at 54 °C (for streptococci), and 1 min at 72 °C with a final extension step of 10 min at 72 °C. The 933-bp (staphylococci) and 945-bp (streptococci) amplicons were examined by electrophoresis in 1% agarose gels, stained with Sybr Safe DNA gel stain (Invitrogen, CA, USA), and visualized under a UV transilluminator.

Restriction fragment length polymorphism (RFLP) analysis

Fifteen microliters of both PCR amplifications were digested in a 30 µL volume containing 10×FastDigest Green buffer, 0.25 µL of 20 mg/mL acetylated BSA, and 1 µL of FastDigest *Alu*I enzyme (Thermo Scientific, CA, USA). Reaction mixtures were incubated at 37 °C for 15 min and directly loaded on the precast gels. Twenty microliters of digested *gap* amplifications were loaded in 12% Bis-Tris NuPAGE™ gels (Invitrogen and then electrophoresed in a vertical gel apparatus. For staphylococci, the following 14 T/RS were used as reference strains for restriction pattern comparison: *Staph. epidermidis* ATCC 35983, *Staph. xylosus* ATCC 29971 ^{T}, *Staph. saprophyticus* ATCC 15305 ^{T}, *Staph. capitis* ATCC 27840 ^{T}, *Staph. haemolyticus* ATCC 29970 ^{T}, *Staph. simulans* ATCC 27848 ^{T}, *Staph. warneri* ATCC 27836 T, *Staph. arlettae* ATCC 43957 ^{T}, *Staph. chromogenes* ATCC 43764 ^{T}, *Staph. equorum* ATCC 43958 ^{T}, *Staph. caprae* ATCC 35538 ^{T}, *Staph. sciuri* ATCC 29062 ^{T}, *Staph. hiycus* ATCC 11249 ^{T}, and *Staph. intermedius* ATCC 29663 ^{T}. The *gap* PCR–RFLP patterns used for *Staphylococcus* species assignment are illustrated in Additional file 3. For streptococci, the following 7 T/RS were used as reference strains for restriction pattern comparison: *Strep. uberis* ATCC 700407, *Strep. dysgalactiae* subsp. *dysgalactiae* ATCC 43078 ^{T}, *Strep. dysgalactiae* subsp. *equisimilis* DSM 23147 ^{T}, *Strep. agalactiae* ATCC 13813 ^{T}, *Strep. gallolyticus*

subsp. *gallolyticus* ATCC 49475, *Strep. equi* subsp. *zooepidermicus* NCTC 6180, and *Strep. suis* ATCC 43765. The *gap* PCR–RFLP patterns used for *Streptococcus* species assignment are illustrated in Additional file 4. MALDI-TOF MS confirmed all reference strain species identities.

Amplicon sequencing

The *gap* gene amplicons of all isolates with PCR–RFLP profiles different from those of *Staphylococcus* and *Streptococcus* T/RS were sequenced at BMR Genomics with the Sanger sequencing option. The nucleotide sequences were compared to sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST).

Comparison of PCR–RFLP and MALDI-TOF MS results

All the data related to MALDI-TOF MS results, log scores, PCR–RFLP identification, *gap* gene sequencing results, and animal species were plotted with Microsoft Excel for calculations of agreement ratio (AR), score distribution, and relative percentage values. Plots were generated with Microsoft Excel.

Results

Isolates and geographical distribution

From January 2021 to May 2022, 204 NASM and 57 streptococci, for a total of 261 isolates, were obtained from sheep and goat milk samples sent to the IZSSA for microbiological analysis with a diagnosis of clinical mastitis/visible milk alterations by the farm veterinarian. Ovine isolates were 246, of which 191 were NASM and 55 streptococci. Caprine isolates were 15, of which 13 NASM and two streptococci (Additional file 5).

MALDI-TOF MS of NASM isolates

All 204 isolates typed as NASM by culture, and preliminary biochemical tests were subjected to MALDI-TOF MS identification by processing two spots per colony with the direct transfer procedure after 24 h of bacterial growth. This enabled the successful species identification of 201 isolates (98.5%), with log scores ≥ 2.00 in 165 (80.9%) and between 1.70–1.99 in 36 (17.6%). For the remaining three isolates (1.5%), log scores were <1.70, with no identification possible even after a second round of identification with the direct transfer, extended direct transfer, and protein extraction procedures (Table 1). The most frequently identified species were *Staph. epidermidis* (59, 28.9%), *Staph. chromogenes* (57, 27.9%), *Staph. haemolyticus* (32, 15.7%), *Staph. caprae* (13, 6.4%), and

Staph. simulans (13, 6.4%), accounting for 85.3% of all NASM isolates. The remaining 13.2% was represented by *Staph. microti* (6, 2.9%), *Staph. xylosus* (4, 2.0%), *Staph. equorum*, *petrasii, and warneri* (3 each, 1.5%), *Staph. sciuri* (now *M. sciuri*) (2, 1.0%), and *Staph. arlettae*, *capitis*, *cohnii, lentus* (now *M. lentus*), *pseudintermedius*, and *succinus* (1 each, 0.5%).

MALDI-TOF MS of streptococcus isolates

MALDI-TOF MS identification of the 57 streptococcus isolates included in this study enabled the successful species identification of all isolates (100%) with log scores ≥2.00 in 53 (93.0%) and between 1.7–1.99 in 4 (7.0%). The most frequently identified species was *Strep. uberis* (51, 89.5%), followed by *Strep. dysgalactiae* and *parauberis* (2 each, 3.5%), and by *Strep. gallolyticus* and *suis* (1 each, 1.8%) (Table 1).

PCR–RFLP of NASM isolates

All 204 isolates typed as NASM by culture, and preliminary biochemical tests were also subjected to species identification by *gap* PCR–RFLP. A total of 187 (91.7%) isolates showed restriction profiles identical to the reference strains, while 17 (8.3%) isolates showed a different PCR–RFLP pattern (Table 1). Upon *gap* gene sequencing (Additional file 6), 12 were identified as *Staph. chromogenes* (1), *devriesei* (1), *epidermidis* (1), *haemolyticus* (3), *hyicus* (1), *jettensis* (1), *muscae* (1), *pseudintermedius* (1), *pseudoxylosus* (1), and *simulans* (1), while five remaining isolates were classified as *Staph. muscae* (4) and *Staph. devriesei* (1) by matching with the isolate classified by *gap* gene sequencing (Table 2).

As a result, the top three identified species were *Staph. epidermidis* (61, 29.9%), *Staph. chromogenes* (58, 28.4%), and *Staph. haemolyticus* (37, 18.1%), followed by *Staph. simulans* (14, 6.9%) *Staph. caprae* (13, 6.4%), *Staph. muscae* (5, 2.5%), *Staph. equorum* and *warneri* (3, 1.5%), *Staph. devriesei* and *xylosus* (2 each, 1.0%), and *Staph. arlettae*, *capitis*, *hyicus*, *jettensis*, *pseudintermedius*, and *pseudoxylosus* (1 each, 0.5%).

PCR–RFLP of streptococcus isolates

Out of the 57 isolates typed as *Streptococcus* sp. By culture and preliminary biochemical tests, 53 (93.0%) showed *gap* PCR–RFLP profiles identical to the reference strains, while 4 (7.0%) did not. Upon *gap* gene sequencing (Additional file 7), these were identified as *Strep. parauberis* (2), *Strep. Uberis* (1), and *Strep. ruminantium* (1) (Table 1 and Table 2). As a result, the top identified species

was *Strep. uberis* (51, 89.5%), followed by *Strep. dysgalactiae* and *parauberis* (2 each, 3.5%), and by *Strep. gallolyticus* and *ruminantium* (1 each, 1.8%).

Comparison of gap PCR–RFLP and MALDI-TOF MS results

The species identification results obtained by MALDI- TOF MS and *gap* PCR–RFLP were in general agreement, as detailed in Table 1 and illustrated in Figure 1. For the five most frequently identified NAS (*Staph. epidermidis*, *chromogenes*, *haemolyticus*, *caprae*, and *simulans*), the agreement rate (AR) between MALDI-TOF MS and *gap* PCR–RFLP was 100%. Log scores were mostly ≥2.00, although for *Staph. haemolyticus* only 56.3% of the identifications had log scores ≥ 2.00. The AR between the two identification methods was 100% also for *Staph. equorum*, *warneri*, *arlettae*, *capitis*, and *pseudintermedius*., AR was 50% for *Staph. xylosus* and 0% for *Staph. microti*, *Staph. petrasii*, *Staph. sciuri* (now *M. sciuri)*, *Staph. cohnii*, *Staph. lentus* (now *M. lentus*), and *Staph. succinus* (Table 1).

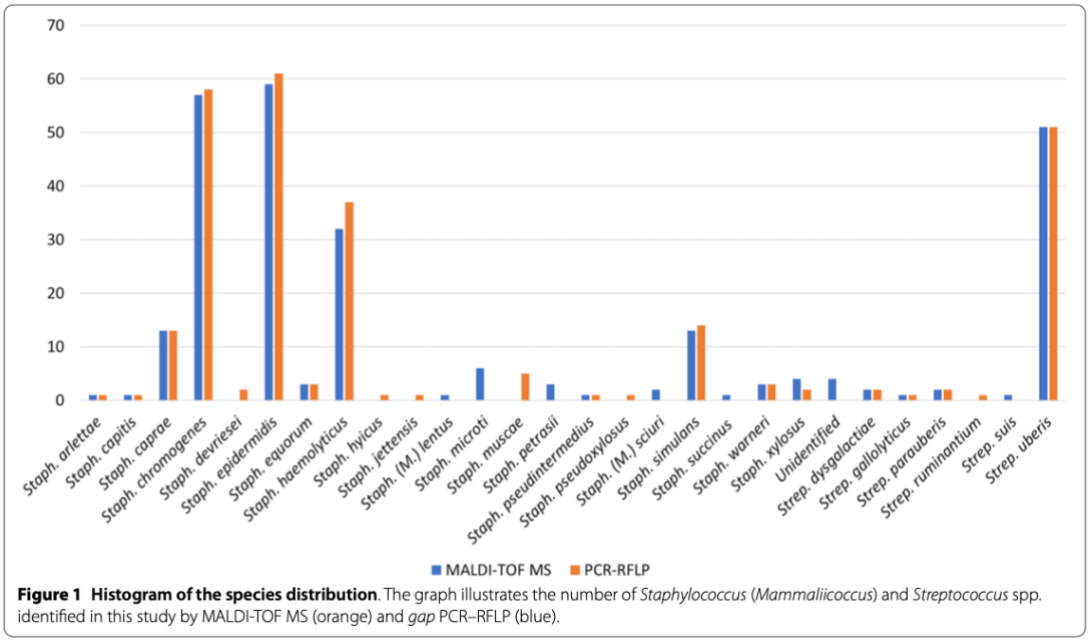

Figure 1 Histogram of the species distribution. The graph illustrates the number of *Staphylococcus* (*Mammaliicoccus*) and *Streptococcus* spp. identified in this study by MALDI-TOF MS (orange) and *gap* PCR–RFLP (blue).

Table 1 Summary of species identification results obtained by MALDI-TOF MS and *gap* PCR–RFLP on the 261 isolates evaluated in this study

Species ID by MALDI-TOF MS[a]	N. of isolates[b]	Log score			AR with *gap* PCR-RFLP[f]
		≥ 2.0[c]	1.7–1.99[d]	< 1.7 (no ID)[e]	
Staphylococci					
Staphylococcus epidermidis	59 (28.9%)	55 (93.2%)	4 (6.8%)	–	59 (100%)[g]
Staphylococcus chromogenes	57 (27.9%)	53 (93.0%)	4 (7.0%)	–	57 (100%)[g]
Staphylococcus haemolyticus	32 (15.7%)	18 (56.3%)	14 (43.7%)	–	32 (100%)[g]
Staphylococcus caprae	13 (6.4%)	10 (76.9%)	3 (23.1%)	–	13 (100%)[g]
Staphylococcus simulans	13 (6.4%)	13 (100%)	–	–	13 (100%)[g]
Staphylococcus microti	6 (2.9%)	3 (50%)	3 (50%)	–	0 (0%)
Staphylococcus xylosus	4 (2.0%)[8]	2 (50%)	2 (50%)	–	2 (50%)[g]
Staphylococcus equorum	3 (1.5%)	3 (100%)	–	–	3 (100%)[g]
Staphylococcus petrasii	3 (1.5%)	3 (100%)	–	–	0 (0%)
Staphylococcus warneri	3 (1.5%)	2 (66.7%)	1 (33.3%)	–	3 (100%)[g]
Staphylococcus sciuri[h]	2 (1.0%)	2 (100%)	–	–	0 (0%)[g]
Staphylococcus arlettae	1 (0.5%)	–	1 (100%)	–	1 (100%)[g]
Staphylococcus capitis	1 (0.5%)	1 (100%)	–	–	1 (100%)[g]
Staphylococcus cohnii	1 (0.5%)	–	1 (100%)		0 (0%)
Staphylococcus lentus[h]	1 (0.5%)[9]	–	1 (100%)	–	0 (0%)
Staphylococcus pseudintermedius	1 (0.5%)	–	1 (100%)	–	1 (100%)[g]
Staphylococcus succinus	1 (0.5%)	–	1 (100%)	–	0 (0%)
Unidentified	3 (1.5%)	–	–	3 (100%)	–
Total staphylococci	204	165 (80.9%)	36 (17.6%)	3 (1.5%)	185 (90.7%)
Streptococci					
Streptococcus uberis	51 (89.5%)	48 (94.1%)	3 (5.9%)	–	51 (100%)[g]
Streptococcus dysgalactiae[910]	2 (3.5%)	2 (100%)	–	–	2 (100%)[g]
Streptococcus parauberis	2 (3.5%)	2 (100%)	–	–	2 (100%)[k]
Streptococcus gallolyticus	1 (1.8%)	1 (100%)	–	–	1 (100%)[g]
Streptococcus suis	1 (1.8%)	–	1 (100%)	–	0 (0%)[g]
Unidentified	0 (1.8%)	–	–	–	–
Total streptococci	57	53 (93.0%)	4 (7.0%)	0 (0%)	56 (98.2%)

[a] Based on the Bruker MALDI BioTyper System Compass® Library Revision H (2021), covering 3893 species/entries.

[B] Percent data represent the proportion of a given species isolated among all staphylococci or streptococci (within the column).

[C] Percent data represent the proportion of isolates identified with MALDI log scores ≥ 2.0 among all isolates of the same species.

[D] Percent data represent the proportion of isolates identified with MALDI log scores 1.7–1.99 among all isolates of the same species.

[E] Percent data represent the proportion of isolates with MALDI log scores < 1.7 among all isolates of the same species and no identification possible (no ID).

[F] Number of isolates identified as the same species by MALDI-TOF MS and *gap* PCR–RFLP. Percent data represent the proportion of isolates identified as the same species (Agreement Rate, AR).

[G] The reference strain for this species was included in the *gap* PCR–RFLP identification panel (please see the materials and methods section for the reference isolate list).

[H] These species have been reclassified in the *Mammaliicoccus* genus (Madhaiyan et al., 2020).

[I] For one isolate, the first round of MALDI-TOF MS identification was unsuccessful and repeated.

[J] *Streptococcus dysgalactiae* and *Streptococcus canis* cannot be resolved by MALDI-TOF MS.

[K] Both assigned by *gap* gene amplicon sequencing and alignment with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Details are reported in Additional file 7.

The discordant species identifications between the two approaches are reported in Table 2. Of note was the case of 6 isolates identified as *Staph. microti* by MALDI-TOF MS and as *Staph. muscae* (5 isolates) and *Staph. simulans* (1 isolate) by *gap* sequencing. Only gap sequencing was provided to identify Staph among less frequently identified NASM species. *muscae*, *devriesei*, *hyicus*, *jettensis*, and *pseudoxylosus*, while only MALDI-TOF MS provided *Staph. microti, Staph. petrasii, Staph. sciuri* (now *M. sciuri)*, *Staph. lentus* (now *M. lentus),* and *Staph. succinus*. For streptococci, the AR between MALDI-TOF MS and *gap* PCR–RFLP was 98.2% (56 of 57). The AR was 100% for the identification of *Strep. uberis*, with log scores≥2.00 in 94.1% of cases. The AR was also 100% for *Strep. dysgalactiae*, *parauberis*, and *gallolyticus*. Only one species was identified as *Strep. suis* by MALDI-TOF MS (log score 1.78) and as *Strep. ruminantium* by *gap* sequencing (Table 2).

Table 2 Detail of the 20 discordant species identification results between MALDI-TOF MS and *gap* PCR–RFLP integrated with *gap* gene sequencing

MALDI-TOF MS[a]	Log score	ID by *gap* PCR-RFLP[b]	ID by *gap* sequencing[c] (% identity)[d]
Staphylococci			
Staphylococcus microti	1.99	–	*Staphylococcus muscae* (92.79%)
Staphylococcus microti	2.05	–	*Staphylococcus muscae*[e]
Staphylococcus microti	2.06	–	*Staphylococcus muscae*[e]
Staphylococcus microti	1.91	–	*Staphylococcus muscae*[e]
Staphylococcus microti	1.76	–	*Staphylococcus muscae*[e]
Staphylococcus microti	2.04	–	*Staphylococcus simulans* (99.89%)
Staphylococcus petrasii	2.1	*Staphylococcus haemolyticus*	–
Staphylococcus petrasii	2.03	*Staphylococcus haemolyticus*	–
Staphylococcus petrasii	2.24	–	*Staphylococcus jettensis* (99.29%)
Staphylococcus sciuri[f]	2.23	–	*Staphylococcus chromogenes* (98.75%)
Staphylococcus sciuri[f]	2.16	–	*Staphylococcus hyicus* (98.69%)
Staphylococcus xylosus	1.88	–	*Staphylococcus devriesei*[g]
Staphylococcus xylosus	1.81[g]	–	*Staphylococcus pseudoxylosus* (99.78%)
Staphylococcus cohnii	1.7[h]	–	*Staphylococcus haemolyticus* (98.91%)
Staphylococcus lentus[f]	1.8[h]	–	*Staphylococcus epidermidis* (98.46%)
Staphylococcus succinus	1.93	*Staphylococcus epidermidis*	–
Unidentified	1.6	–	*Staphylococcus haemolyticus* (99.07%)
Unidentified	1.28	–	*Staphylococcus haemolyticus* (98.26%)
Unidentified	1.44	–	*Staphylococcus devriesei* (99.77%)
Streptococci			
Streptococcus suis	1.78	–	*Streptococcus ruminantium* (98.15%)

[a] Based on the Bruker MALDI BioTyper System Compass® Library Revision H (2021), covering 3893 species/entries.
[b] Species identification was assigned by matching the enzyme digestion pattern of the gap gene amplicon with the reference strain.
[c] All amplicons producing an enzyme digestion pattern different than the reference strains were subjected to genomic sequencing for species identification.
[d] Percent identity of the gap gene amplicon with sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Details are reported in Additional files 6 and 7.
[e] Based on the *Staphylococcus muscae* isolate restriction pattern identified by gap sequencing.
[f] These species have been reclassified in the *Mammaliicoccus* genus (Madhaiyan et al., 2020).
[g] Based on the restriction pattern of the *Staphylococcus devriesei* isolate identified by gap sequencing.
[h] The first round of MALDI-TOF MS identification was unsuccessful and repeated.

Discussion

NAS and streptococci are the most prevalent etiological agents of small ruminant mastitis (Gelasakis et al., 2015; Dore et al., 2016). NAS, mammaliicocci, and streptococci can also be found in milk as contaminants and components of the mammary gland microbiota (Madhaiyan et al., 2020; Oikonomou et al., 2020; Derakhshani et al., 2018; Addis et al., 2016). Obtaining a reliable species identification is essential for understanding their epidemiology and roles in mammary gland health and disease to make more meaningful management decisions (Bernier Gosselin et al., 2019; De Buck et al., 2021; Gosselin et al., 2018). In the last decade, MALDI-TOF MS has emerged as a dependable method for microbial identification and is being increasingly applied to bacteria and fungi isolated from bovine milk. When MALDI-TOF MS instrumentation is available in the laboratory, this approach is rapid, cost-effective, high-throughput, reliable, and does not require specific knowledge of mass spectrometry or molecular biology. An isolated colony is sufficient for identification, and results are available within minutes. Thanks to the short analytical times, many targets can be processed during the day, making it possible to identify thousands of microbial isolates. A further advantage of MALDI-TOF MS is the possibility of creating personalized spectrum libraries, including isolates of specific interest, improving bacterial identification reliability (Kostrzewa et al., 2016). In the last decade, the IZSSA has developed and applied in its diagnostic routine a *gap* PCR–RFLP method enabling the identification of NAS and streptococci isolated from small ruminant milk (Onni et al., 2019; Onni et al., 2012; Rosa et al., 2019). This approach is reliable but moderately expensive in terms of materials and labor as it requires DNA extraction, PCR amplification, amplicon digestion, electrophoresis, and result interpretation by comparison with RFLP profiles from reference strains. The restricted number of reference strains is also a limitation, and it may eventually require *gap* gene amplicon sequencing for a tentative identification by homology. *Gap* PCR–RFLP may represent a valuable alternative when MALDI-TOF instrumentation is unavailable. Despite the low cost per test, the mass spectrometer acquisition costs are too high for many veterinary diagnostic facilities, especially in low-resource contexts or in peripheral laboratories. As a further consideration, scarce data are available in the literature on applying MALDI-TOF MS identification to the microorganisms isolated from small ruminant milk, especially concerning NAS and sheep. In this work, we identified NASM and streptococci isolated from the milk of small ruminants with mastitis by MALDI-TOF MS and *gap* PCR–RFLP integrated with amplicon sequencing. As a result, the species most frequently identified in our study were in line with those reported as causing small ruminant IMI worldwide (Vasileiou et al., 2019; Vanderhaeghen et al., 2014), that is, *Staph. epidermidis*, *chromogenes*, *haemolyticus*, *caprae,* and *simulans* for NAS and *Strep. uberis* for streptococci. Notably, the two identification approaches showed a very high level of agreement, even

for MALDI-TOF MS identifications with log scores ≥1.7. A cutoff log score ≥1.7 for species identification has been validated by various authors (Conesa et al., 2020; Mahmmod et al., 2018; Han et al., 2015) as highly appropriate and accurate for bovine NAS. Han et al. (Han et al., 2015) found that the ≥ 1.7 log score threshold enabled reaching a significantly higher level of NAS species identification without sacrificing specificity. Cameron et al. (Cameron et al., 2018) also found that the reduction of the species level cutoff improved method performance from 64% to 92% when classifying bovine-associated NAS isolates. In the more recent study by Conesa et al. (Pain et al., 2020), the ≥1.7 score made it possible to successfully identify 36 more strains, as validated by the comparison with genotypic methods. Based on our results, a log score threshold of≥1.7 could be considered reliable also for the most prevalent small ruminant NAS. For *Staph. haemolyticus*, the log scores were generally lower, as 43.7% of isolates had values <2.0. Notably, 2 isolates were identified as *Staph. haemolyticus* by PCR–RFLP and as *Staph. petrasii* by MALDI-TOF MS with log scores > 2.0. The *gap* gene of three isolates showed a very high sequence identity to *Staph. haemolyticus,* but these were either identified as *Staph. cohnii* with a very low log score (1.7) or were not identified by MALDI-TOF MS. In our routine work, *Staph. haemolyticus* isolated from cow milk is also typically identified with lower scores among the most prevalent *Staphylococcus* spp. (M.F.A., personal communication). *Staph. haemolyticus* may be more problematic to identify because of a higher genomic variability and the similarity of marker genes with other species. Wanecka et al. (Wanecka et al., 2019) found that 27 of 33 of their *Staph. haemolyticus* isolates had 99.5–100% similarity of the 16S rRNA gene with *Staph. petrasii* subsp. *jettensis* (now *Staph. petrasii* subsp. *petrasii*), *Staph. hominis*, *Staph. epidermidis*, or *Staph. devriesei*. Also, some *Staph. haemolyticus* have been reclassified as *Staph. borealis* (Pain et al., 2020). Molecular techniques may have limitations, including insufficient discriminatory power in the case of closely related species or the lack of quality of sequences deposited in the GenBank (Heikens et al., 2005), as also observed in this work for *gap* gene sequencing. The absence of reference spectra for these highly similar minor species in the MALDI-TOF MS database might be the reason for their identification as *Staph. haemolyticus* with lower log scores. All six isolates were identified as *Staph. microti* by MALDI-TOF MS could not be identified by *gap* PCR–RFLP. The *gap* gene sequence showed the highest identity with *Staph. muscae* (5 out of 6) and *Staph. simulans* (1 out of 6). *Staph. microti* was described for the first time by Novàkovà et al. in 2010 (Nováková et al., 2010) as an isolate from *Microtus arvalis*, the common vole, with *Staph. muscae* as the nearest relative. Its report as a staphylococcal species associated with mastitis in bovine and bubaline cows is increasing in association with the implementation of MALDI-TOF MS for NAS identification (Hamel et al., 2020, Król et al., 2016, Addis et al., 2022). In consideration of the high similarity between these species and other closely related species not

included in the Bruker spectrum library, such as *Staph. rostri*, this requires further evaluation, possibly followed by spectrum library integration. An isolate identified as *Staph. petrasii* by MALDI-TOF MS with a log score ≥2.00 was identified as *Staph. jettensis* upon *gap* gene sequencing. The species description of *Staph. petrasii* has been emended, and *Staph. jettensis* should be reclassified as a novel subspecies within *Staph. petrasii,* for which the name *Staph. petrasii* subsp. *jettensis* subsp. Nov (De Bel et al., 2014). The identification of this NAS as *Staph. jettensis* by *gap* gene sequencing may have resulted from matching GenBank sequences that were not updated following taxonomic reclassification. In 2020, five NAS species were proposed to be reassigned to the novel *mammaliicoccus* genus, including *Staph. sciuri* and *Staph. lentus*, with *M. sciuri* as the type species (Madhaiyan et al., 2020). The same authors proposed the reclassification of *Staph*. *cohnii subsp*. urealyticus as the novel species *Staph. urealyticus*. The *Mammaliicoccus* genus is not included in the current release of the Bruker library. This should be considered when MALDI-TOF MS identifies these species with the MBT System and the commercial spectrum library release available at the time of this study. Concerning the two isolates identified as *Staph. devriesei* by *gap* gene sequencing and PCR–RFLP, one was not identified by MALDI-TOF MS, while the other was identified as *Staph. xylosus* with a score < 2.00. One isolate identified as *Staph. hyicus* by *gap* gene sequencing was identified as *Staph. sciuri* by MALDI-TOF MS with score ≥2.00. *Staph. devriesei* falls in the *Staph. haemolyticus* group (Vasileiou et al., 2019), and *Staph. sciuri* is also reported as belonging to a separate group from *Staph. hyicus*. *Staph. hyicus* can be difficult to differentiate from *Staph. agnetis* (Adkins et al., 2017), and the latter species was not present in the MALDI-TOF MS spectrum database. An isolate was identified as *Staph. xylosus* by MALDI-TOF MS after a second identification round with a score of 1.81. The same isolate was identified as *Staph. pseudoxylosus* by gap gene sequencing; this species was not included in the spectrum library. Analogously, no *Strep. ruminantium* spectra were present. For this latter species, a presumptive identification as *Strep. suis* was provided with a score ≥1.7. *Strep. ruminantium* is a new species of the *suis* group. *Strep. suis* includes 35 serotypes, of which six have been re-classified to other bacterial species (Gottschalk et al., 2020). Among them, *Strep. suis* serotype 33 has been recently classified as a new species, *Strep. ruminantium* (Tohya et al., 2018), of which the reference strain was originally isolated from a lamb. As the two species are biochemically very similar, differentiation is difficult and a PCR specific for *Strep. ruminantium* has recently been described (Okura et al., 2019). All other streptococci showed a very high agreement rate; both MALDI-TOF MS and PCR–RFLP overcome the known difficulties in the identification of Gram-positive, catalase-negative cocci by biochemical reactions (Scillieri et al., 2020; Fortin et al., 2003) also with streptococci isolated from small ruminant milk. We carried out microbial cultivation and target preparation at the IZSSA in Sassari, Sardinia, and MALDI-TOF

MS identification at the University of Milan in Lodi, Lombardy, on the following day. According to the Bruker MBT User Manual, result reliability is maintained if spectra are generated within 24 h from target preparation. This opens the possibility that these may be prepared in one laboratory and sent to a shared core facility for identification within the following day, provided that convenient logistic solutions are in place. This approach would constrain instrumental costs while centralizing the mass spectrometry instrumentation and the spectrum library. This might also represent a reasonable alternative to the *gap* gene sequencing to integrate the PCR–RFLP identification approach. In planning such a setup, it would be advisable to thoroughly assess the impact of target transportation temperatures and conditions on the reliability of the MALDI-TOF MS identification results. Our results concerning the species of NASM and streptococci associated with sheep and goat mastitis were in line with those reported as causing small ruminant IMI worldwide (Vasileiou et al., 2019; Vanderhaeghen et al., 2014). MALDI-TOF MS and gap PCR–RFLP provided comparable results for the most prevalent species. *Gap* PCR–RFLP can offer a reliable identification alternative when MALDI-TOF MS is unavailable. Restriction profiles differing from the validated reference isolates (Onni et al., 2010, Onni et al., 2012, Rosa et al., 2019) may not be easily resolved by *gap* gene sequencing. Concerning MALDI-TOF MS, integrating the spectrum library with small ruminant strains of *Staph. haemolyticus* as well as of *Staph. microti* and their closely related species might further improve identification performances.

Supplementary Materials

The online version contains supplementary material available at https://doi. Org/10.1186/s13567-022-01102-4.

Additional file 1: Geographical distribution of all 261 isolates included in this study.

Additional file 2: Staphylococcus and Streptococcus species included in the MBT Compass Library Revision H (2021).

Additional file 3: Restriction fragment length polymorphism (RFLP) pattern of PCR products of the *gap* gene obtained after digestion with *Alu*I and used for *Staphylococcus* species assignment.

Additional file 4: Restriction fragment length polymorphism (RFLP) pattern of PCR products of the *gap* gene obtained after digestion with *Alu*I and used for *Streptococcus* species assignment.

Additional file 5: Excel file detailing PCR–RFLP identification, *gap* gene sequencing information where obtained, MALDI-TOF MS identification, best log score, and animal species.

Additional file 6: Genomic sequence of the *gap* gene and sequence similarity data for staphylococci.

Additional file 7: Genomic sequence of the *gap* gene and sequence similarity data for streptococci.

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Competing interests

The authors declare that they have no competing interests.

Authors' contributions

NMR, MP, and SF-P performed the laboratory experiments. MFA and ST designed the study, analyzed the data, and wrote the manuscript. All authors read and approved the final manuscript.

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References

Addis MF, Maffioli EM, Penati M, Albertini M, Bronzo V, Piccinini R, Tangorra F, Tedeschi G, Cappelli G, Di Vuolo G, Vecchio D, De Carlo E, Ceciliani F (2022) Peptidomic changes in the milk of water buffaloes (*Bubalus bubalis*) with intramammary infection by non-aureus staphylococci. Sci Rep 12:8371. https://doi.org/10.1038/s41598-022-12297-z

Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P (2016) The bovine milk microbiota: insights and perspectives from -omics studies. Mol Biosyst 12:2359–2372. https://doi.org/10.1039/C6MB00217J

Adkins PRF, Middleton JR, Calcutt MJ, Stewart GC, Fox LK (2017) Species identification and strain typing of *Staphylococcus agnetis* and *Staphylo- coccus hyicus* isolates from bovine milk by use of a novel multiplex PCR assay and pulsed-field gel electrophoresis. J Clin Microbiol 55:1778–1788. https://doi.org/10.1128/JCM.02239-16

Bernier Gosselin V, Dufour S, Calcutt MJ, Adkins PRF, Middleton JR (2019) Staphylococcal intramammary infection dynamics and the relationship with milk quality parameters in dairy

goats over the dry period. J Dairy Sci 102:4332–4340. https://doi.org/10.3168/JDS.2018-16030

Cameron M, Perry J, Middleton JR, Chaffer M, Lewis J, Keefe GP (2018) Short communication: evaluation of MALDI-TOF mass spectrometry and a custom reference spectra expanded database for the identification of bovine-associated coagulase-negative staphylococci. J Dairy Sci 101:590– 595. https://doi.org/10.3168/jds.2017-13226

Conesa A, Dieser S, Barberis C, Bonetto C, Lasagno M, Vay C, Odierno L, Porporatto C, Raspanti C (2020) Differentiation of non-*aureus* staphylococci species isolated from bovine mastitis by PCR-RFLP of groEL and gap genes in comparison to MALDI-TOF mass spectrometry. Microb Pathog 149:104489. https://doi.org/10.1016/j.micpath.2020.104489

Conington J, Cao G, Stott A, Bünger L (2008) Breeding for resistance to mastitis in United Kingdom sheep, a review and economic appraisal. Vet Rec 162:369–376. https://doi.org/10.1136/VR.162.12.369

De Bel A, Švec P, Petráš P, Sedláček I, Pantůček R, Echahidi F, Vandamme DP (2014) Reclassification of *Staphylococcus jettensis* De Bel, et al 2013 as *Staphylococcus petrasii* subsp. Jettensis subsp. Nov. and emended description of *Staphylococcus petrasii* Pantucek et al. 2013. Int J Syst Evol Microbiol 64:4198–4201. https://doi.org/10.1099/ijs.0.064758-0

De Buck J, Ha V, Naushad S, Nobrega DB, Luby C, Middleton JR, De Vliegher S, Barkema HW (2021) Non-*aureus* staphylococci and bovine udder health: current understanding and knowledge gaps. Front Vet Sci 8:360. https://doi.org/10.3389/fvets.2021.658031

Derakhshani H, Fehr KB, Sepehri S, Francoz D, De Buck J, Barkema HW, Plaizier JC, Khafipour E (2018) Invited review: microbiota of the bovine udder: contributing factors and potential implications for udder health and mastitis susceptibility. J Dairy Sci 101:10605–10625. https://doi.org/ 10.3168/jds.2018-14860

Dore S, Liciardi M, Amatiste S, Brgagna S, Bolzoni G, Caligiuri V, Cerrone A, Farina G, Montagna CO, Saletti MA, Scatassa ML, Sotgiu G, Cannas EA (2016) Survey on small ruminant bacterial mastitis in Italy, 2013–2014. Small Rumin Res 141:91–93. https://doi.org/10.1016/j.smallrumres.2016. 07.010

Fortin M, Messier S, Paré J, Higgins R (2003) Identification of catalase- negative, non-beta-hemolytic, Gram-positive cocci isolated from milk samples. J Clin Microbiol 41:106–109

Gelasakis AI, Mavrogianni VS, Petridis IG, Vasileiou NGC, Fthenakis GC (2015) Mastitis in sheep—the last 10 years and the future of research. Vet Microbiol 181:136–146. https://doi.org/10.1016/j.vetmic.2015.07.009

Gosselin VB, Lovstad J, Dufour S, Adkins PRF, Middleton JR (2018) Use of MALDI-TOF to characterize staphylococcal intramammary infections in dairy goats. J Dairy Sci 101:6262–6270. https://doi.org/10.3168/jds. 2017-14224

Gottschalk M, Lacouture S, Fecteau G, Desrochers A, Boa A, Saab ME, Okura M (2020) Canada: Isolation of *Streptococcus ruminantium* (*Streptococcus suis*-like) from diseased ruminants in Canada. Can Vet J 61:473

Hamel J, Zhang Y, Wente N, Krömker V (2020) Non-S aureus staphylo- cocci (NAS) in milk samples: Infection or contamination? Vet Microbiol 242:108594. https://doi.org/10.1016/j.vetmic.2020.108594

Han HW, Chang HC, Hunag AH, Chang TC (2015) Optimization of the score cutoff value for routine identification of *Staphylococcus* species by matrix-assisted laser desorption ionization-time-of-flight mass spectrometry. Diagn Microbiol Infect Dis 83:349–354. https://doi.org/10.1016/j. diagmicrobio.2015.08.003

Heikens E, Fleer A, Paauw A, Florijn A, Fluit AC (2005) Comparison of genotypic and phenotypic methods for species-level identification of clinical isolates of coagulase-negative staphylococci. J Clin Microbiol 43:2286–2290. https://doi.org/10.1128/JCM.43.5.2286-2290.2005

Kostrzewa M, Schubert S (2016) MALDI-TOF Mass Spectrometry in Micro- biology. Caister Academic Press, UK

Król J, Wanecka A, Twardoń J, Mrowiec J, Dropińska A, Bania J, Podkowik M, Korzeniowska-Kowal A, Paściak M (2016) Isolation of *Staphylococcus microti* from milk of dairy cows with mastitis. Vet Microbiol 182:163–169. https://doi.org/10.1016/j.vetmic.2015.11.018

Madhaiyan M, Wirth JS, Saravanan VS (2020) Phylogenomic analyses of the *Staphylococcaceae* family suggest the reclassification of five species within the genus *Staphylococcus* as heterotypic synonyms, the promo- tion of five subspecies to novel species, the taxonomic reassignment of five *Staphylococcus* species to *Mammaliicoccus* gen. nov., and the formal assignment of *Nosocomiicoccus* to the family *Staphylococcaceae*. Int J Syst Evol Microbiol 70:5926–5936. https://doi.org/10.1099/ijsem.0.004498

Mahmmod YS, Nonnemann B, Svennesen L, Pedersen K, Klaas IK (2018) Typeability of MALDI-TOF assay for identification of non-*aureus* staphylococci associated with bovine intramammary infections and teat apex colonization. J Dairy Sci 101:9430–9438. https://doi.org/10.3168/jds. 2018-14579

Marogna G, Pilo C, Vidili A, Tola S, Schianchi G, Leori SG (2012) Comparison of clinical findings, microbiological results, and farming parameters in goat herds affected by recurrent infectious

mastitis. Small Rumin Res 102:74–83. https://doi.org/10.1016/J.SMALLRUMRES.2011.08.013

Marogna G, Rolesu S, Lollai S, Tola S, Leori SG (2010) Clinical findings in sheep farms affected by recurrent bacterial mastitis. Small Rumin Res 88:119–125. https://doi.org/10.1016/j.smallrumres.2009.12.019

Nonnemann B, Lyhs U, Svennesen L, Kristensen KA, Klaas IC, Pedersen K (2019) Bovine mastitis bacteria resolved by MALDI-TOF mass spectrometry. J Dairy Sci 102:2515–2524. https://doi.org/10.3168/jds.2018-15424

Nováková D, Pantůček R, Hubálek Z, Falsen E, Busse HJ, Schumann P, Sedláček I (2010) *Staphylococcus microti* sp. Nov., isolated from the com- mon vole (*Microtus arvalis*). Int J Syst Evol Microbiol 60:566–573. https:// doi.org/10.1099/ijs.0.011429-0

Oikonomou G, Addis MF, Chassard C, Nader-Macias MEF, Grant I, Delbès C, Bogni CI, Le Loir Y, Even S (2020) Milk microbiota: what are we exactly talking about? Front Microbiol 11:60. https://doi.org/10.3389/fmicb.2020. 00060

Okura M, Maruyama F, Ota A, Tanaka T, Matoba Y, Osawa A, Sadaat SM, Osaki M, Toyoda A, Ogura Y, Hayashi T, Takamatsu D (2019) Genotypic diversity of Streptococcus suis and the S suis-like bacterium Streptococcus ruminantium in ruminants. Vet Res. https://doi.org/10.1186/ s13567-019-0708-1

Onni T, Sanna G, Cubeddu GP, Marogna G, Lollai S, Leori S, Tola S (2010) Identification of coagulase-negative staphylococci isolated from ovine milk samples by PCR-RFLP of 16S rRNA and gap genes. Vet Microbiol 144:347–352. https://doi.org/10.1016/j.vetmic.2010.01.016

Onni T, Vidili A, Bandino E, Marogna G, Schianchi S, Tola S (2012) Identi- fication of coagulase-negative staphylococci isolated from caprine milk samples by PCR-RFLP of groEL gene. Small Rumin Res 104:185–190. https://doi.org/10.1016/j.smallrumres.2011.10.004

Pain M, Wolden R, Jaén-Luchoro D, Salvà-Serra F, Piñeiro Iglesias B, Karls- son R, Klingenberg C, Cavanagh JP (2020) *Staphylococcus borealis* sp. Nov., isolated from human skin and blood. Int J Syst Evol Microbiol 70:6067– 6068. https://doi.org/10.1099/ijsem.0.004499

Rosa NM, Agnoletti F, Lollai S, Tola S (2019) Comparison of PCR-RFLP, API 20 Strep and MALDI-TOF MS for identification of *Streptococcus* spp. Collected from sheep and goat milk samples. Small Rumin Res 180:35–40. https://doi.org/10.1016/j.smallrumres.2019.09.023

Scillieri Smith JC, Moroni P, Santisteban CG, Rauch BJ, Ospina PS, Nydam VD (2020) Distribution of *Lactococcus* spp. In New York State dairy farms and the association of somatic cell count

resolution and bacteriological cure in clinical mastitis samples. J Dairy Sci 103:1785–1794. https://doi. Org/10.3168/jds.2018-16199

Seng P, Rolain J-M, Fournier PE, La Scola B, Drancourt M, Raoult D (2010) MALDI-TOF-mass spectrometry applications in clinical microbiology. Future Microbiol 5:1733–1754. https://doi.org/10.2217/fmb.10.127

Tohya M, Sekizaki T, Miyoshi-Akiyama T (2018) Complete genome sequence of *Streptococcus ruminantium* sp. Nov. GUT-187T (=DSM 104980T =JCM 31869T), the type strain of *S. ruminantium*, and compari- son with genome sequences of *Streptococcus suis* strains. Genome Biol Evol 10:1180–1184. https://doi.org/10.1093/gbe/evy078

Vanderhaeghen W, Piepers S, Leroy F, Van Coillie E, Haesebrouck F, De Vliegher S (2015) Identification, typing, ecology and epidemiology of coagulase negative staphylococci associated with ruminants. Vet J 203:44–51. https://doi.org/10.1016/j.tvjl.2014.11.001

Vanderhaeghen W, Piepers S, Leroy F, Van Coillie E, Haesebrouck F, De Vliegher S (2014) Invited review: effect, persistence, and virulence of coagulase-negative *Staphylococcus* species associated with ruminant udder health. J Dairy Sci 97:5275–5293. https://doi.org/10.3168/jds. 2013-7775

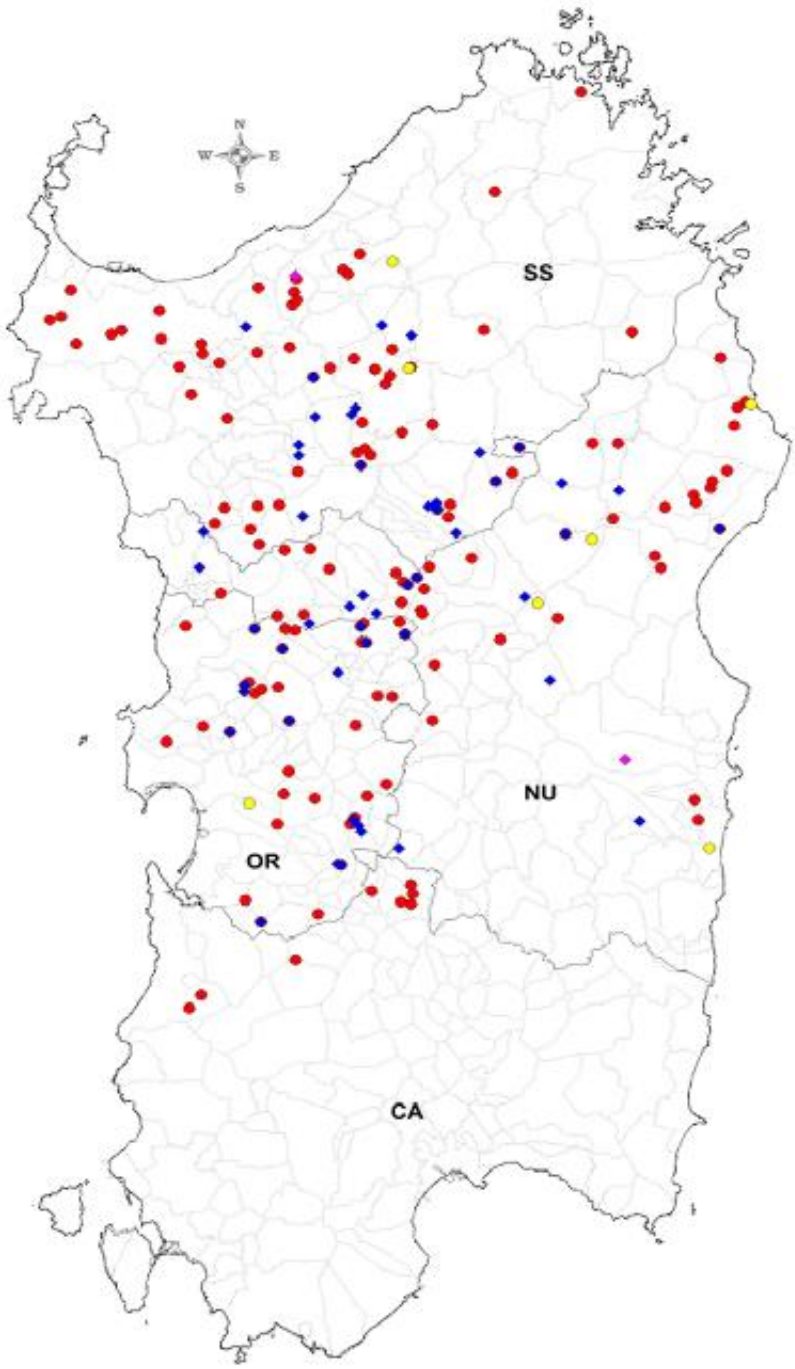
Vasileiou NGC, Chatzopoulos DC, Sarrou S, Fragkou IA, Katsafadou AI, Mavrogianni VS, Petinaki E, Fthenakis GC. Role of staphylococci in mastitis in sheep. J Dairy Res. 2019 Aug;86(3):254-266. Doi: 10.1017/S0022029919000591. Epub 2019 Aug 19. PMID: 31423957.

Wanecka A, Król J, Twardoń J, Mrowiec J, Korzeniowska-Kowal A, Wzorek A (2019) Efficacy of MALDI-TOF mass spectrometry as well as genotypic and phenotypic methods in identification of staphylococci other than Staphylococcus aureus isolated from intramammary infections in dairy cows in Poland. J Vet Diagn Invest 31:523–530. https://doi.org/10.1177/ 1040638719845423

Yugueros J, Temprano A, Berzal B, Sánchez M, Hernanz C, Luengo JM, Naharro G (2000) Glyceraldehyde-3-phosphate dehydrogenase-encoding gene as a useful taxonomic tool for *Staphylococcus* spp. J Clin Microbiol 38:4351–4355. https://doi.org/10.1128/JCM.38.12.4351-4355.2000

Appendix

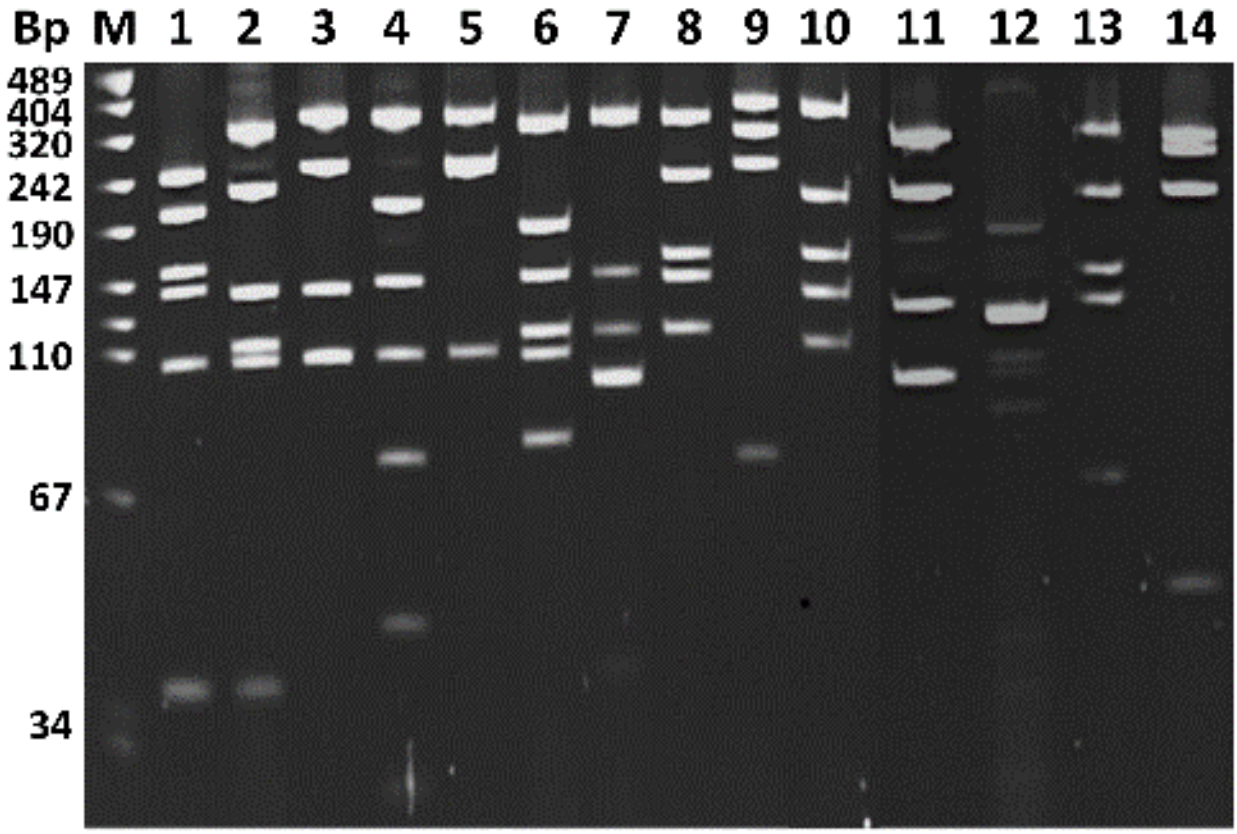
Additional file 1. Geographical distribution of all 261 isolates included in this study. The map reports the location of the 204 non-*aureus Staphylococcus* and *Mammaliicoccus* (NASM), and 57 *Streptococcus* isolates in Sardinia, Italy. Each point represents an individual isolate. Circles indicate NASM: red for sheep milk, and yellow for goat milk. Triangles indicate streptococci: blue for sheep milk, and fuchsia for goat milk.



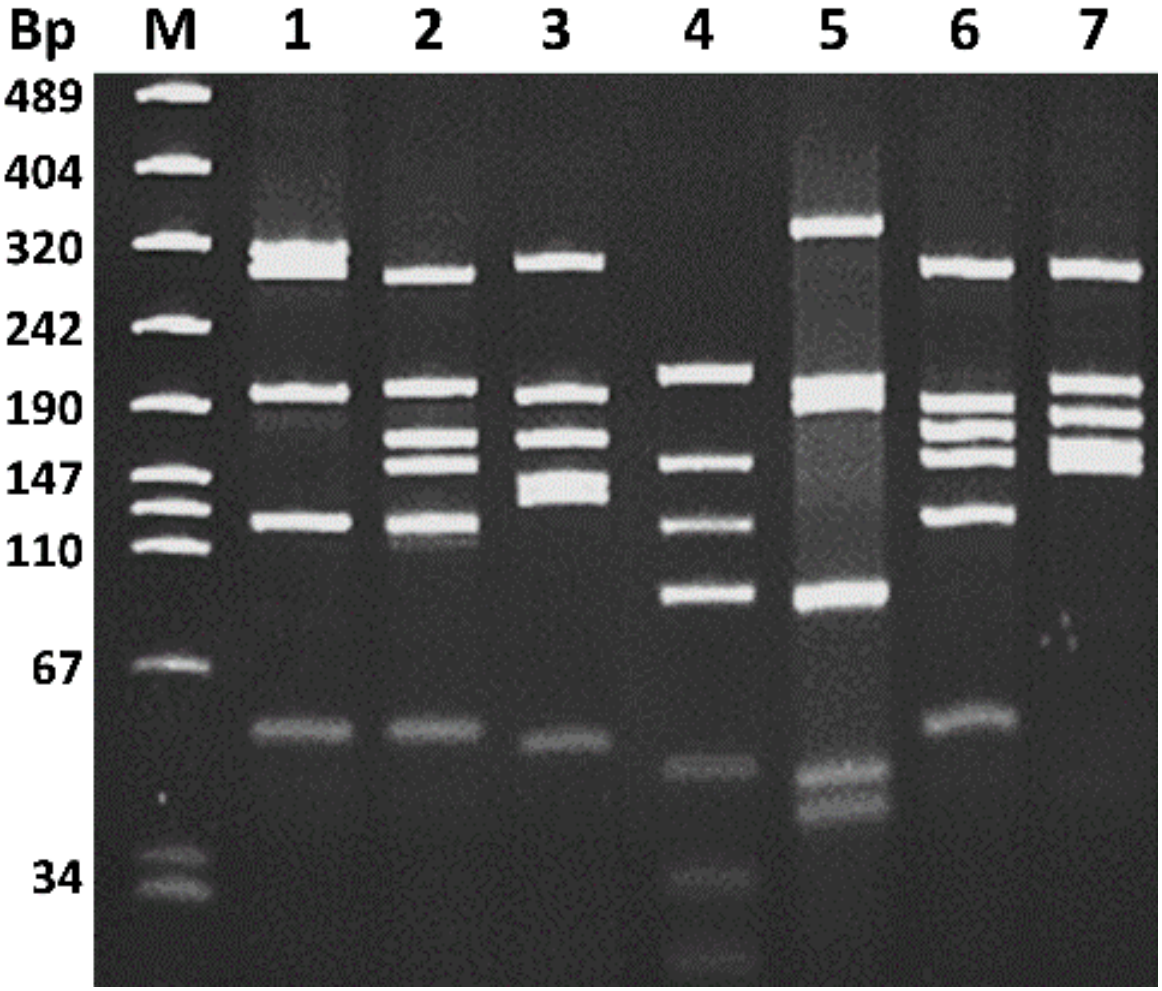
Additional File 2. Staphylococcus and Streptococcus species included in the MBT Compass Library Rev. H (2021), Document Revision E, covering 3893 species/entries.

***Staphylococcus* spp.**	***Streptococcus* spp.**
Staphylococcus argenteus	*Streptococcus acidominimus*
Staphylococcus arlettae	*Streptococcus agalactiae*
Staphylococcus aureus	*Streptococcus alactolyticus*
Staphylococcus auricularis	*Streptococcus anginosus*
Staphylococcus borealis	*Streptococcus australis*
Staphylococcus capitis	*Streptococcus caballi*
Staphylococcus caprae	*Streptococcus canis*
Staphylococcus carnosus	*Streptococcus castoreus*
Staphylococcus chromogenes	*Streptococcus constellatus*
Staphylococcus cohnii	*Streptococcus criceti*
Staphylococcus condimenti	*Streptococcus cristatus*
Staphylococcus delphini	*Streptococcus dentirousetti*
Staphylococcus devriesei	*Streptococcus devriesei*
Staphylococcus epidermidis	*Streptococcus didelphis*
Staphylococcus equorum	*Streptococcus downei*
Staphylococcus felis	*Streptococcus dysgalactiae*
Staphylococcus fleurettii	*Streptococcus entericus*
Staphylococcus gallinarum	*Streptococcus equi_ssp_equi*
Staphylococcus haemolyticus	*Streptococcus equi_ssp_ruminatorum*
Staphylococcus hominis	*Streptococcus equi_ssp_zooepidemicus*
Staphylococcus hyicus	*Streptococcus equinus*
Staphylococcus intermedius	*Streptococcus ferus*
Staphylococcus kloosii	*Streptococcus gallinaceus*
Staphylococcus lentus	*Streptococcus gallolyticus*
Staphylococcus lugdunensis	*Streptococcus gordonii*
Staphylococcus lutrae	*Streptococcus halichoeri*
Staphylococcus massiliensis	*Streptococcus henryi*
Staphylococcus microti	*Streptococcus hyointestinalis*
Staphylococcus muscae	*Streptococcus hyovaginalis*
Staphylococcus nepalensis	*Streptococcus infantarius*
Staphylococcus pasteuri	*Streptococcus infantis*
Staphylococcus petrasii	*Streptococcus iniae*
Staphylococcus pettenkoferi	*Streptococcus intermedius*
Staphylococcus piscifermentans	*Streptococcus lutetiensis*
Staphylococcus pseudintermedius	*Streptococcus macacae*
Staphylococcus saccharolyticus	*Streptococcus marimammalium*
Staphylococcus saprophyticus	*Streptococcus massiliensis*
Staphylococcus schleiferi	*Streptococcus merionis*
Staphylococcus schweitzeri	*Streptococcus minor*
Staphylococcus sciuri	*Streptococcus mitis*
Staphylococcus simiae	*Streptococcus moroccensis*
Staphylococcus simulans	*Streptococcus mutans*
Staphylococcus sp[2]	*Streptococcus oralis*
Staphylococcus stepanovicii	*Streptococcus orisratti*
Staphylococcus succinus	*Streptococcus orisuis*
Staphylococcus vitulinus	*Streptococcus ovis*
Staphylococcus warneri	*Streptococcus parasanguinis*
Staphylococcus xylosus	*Streptococcus parauberis*
	Streptococcus penaeicida
	Streptococcus peroris
	Streptococcus phocae
	Streptococcus pluranimalium
	Streptococcus plurextorum
	Streptococcus pneumoniae
	Streptococcus porci
	Streptococcus porcinus
	Streptococcus porcorum
	Streptococcus pseudopneumoniae
	Streptococcus pseudoporcinus
	Streptococcus pyogenes
	Streptococcus ratti
	Streptococcus salivarius
	Streptococcus salivarius_ssp_thermophilus
	Streptococcus sanguinis
	Streptococcus sinensis
	Streptococcus sobrinus
	Streptococcus sp
	Streptococcus suis
	Streptococcus thoraltensis
	Streptococcus uberis
	Streptococcus urinalis
	Streptococcus vestibularis

Additional file 3. Restriction fragment length polymorphism (RFLP) pattern of PCR products of the *gap* gene obtained after digestion with *Alu*I and used for *Staphylococcus* species assignment. Fragments were separated by 12% NuPAGE gel. Lane 1, *Staphylococcus* (*Staph.*) *epidermidis* ATCC 35983; lane 2, *Staph. xylosus* ATCC 29971^{T}; lane 3, *Staph. saprophyticus* ATCC 15305^{T}; lane 4, *Staph. capitis* ATCC 27840^{T}; lane 5, *Staph. haemolyticus* ATCC 29970^{T}; lane 6, *Staph. simulans* ATCC 27848^{T}; lane 7, *Staph. warneri* ATCC 27836^{T}; lane 8, *Staph. arlettae* ATCC 43957^{T}; lane 9, *Staph. chromogenes* ATCC 43764^{T}; lane 10, *Staph. equorum* ATCC 43958^{T}; lane 11, *Staph. caprae* ATCC 35538^{T}; lane 12, *Staph. sciuri* ATCC 29062^{T}; lane 13, *Staph. hyicus* ATCC 11249^{T}, and lane 14, *Staph. intermedius* ATCC 29663^{T}. M, Marker VIII (Roche).



Additional file 4. Restriction fragment length polymorphism (RFLP) pattern of PCR products of the *gap* gene obtained after digestion with *Alu*I and used for *Streptococcus* species assignment. Fragments were separated by 12% NuPAGE gel. Lane 1, *Streptococcus uberis* ATCC 700407; lane 2, *Strep. dysgalactiae* subsp. *dysgalactiae* ATCC 43078^{T}; lane 3, Strep. *dysgalactiae* subsp. *equisimilis* DSM 23147^{T}; lane 4, *Strep. agalactiae* ATCC 13813^{T}; lane 5, *Strep. gallolyticus* subsp. *gallolyticus* ATCC 49475; lane 6, *Strep. equi* subsp. *zooepidemicus* NCTC 6180; and lane 7, *Strep. suis* ATCC 43765. M, Marker VIII (Roche).



Additional file 5: Excel file detailing PCR–RFLP identification, gap gene sequencing information where obtained, MALDI-TOF MS identification, best log score, and animal species.

Sheet 1: All isolates

Isolate N	MALDI-TOF MS	Log Score	PCR-RFLP (gap gene sequencing identity)
2	Stren. gallolyticus	2,36	Stren. gallolyticus
3	Stren. parauberis	2,17	Stren. parauberis (sequenced, 99.2% *gap*)
4	Stren. dysgalactiae/Stren. canis	2,42	Stren. dysgalactiae
7	Stren. suis	1,78	Stren. ruminantium (sequenced, 97.42% *gap*)
14	Stren. parauberis	2,18	Stren. parauberis (sequenced, 98.67% *gap*)
15	Stren. uberis	2,48	Stren. uberis (sequenced, 99.67% *gap*)
18	Staph. microti	1,99	Staph. muscae (sequenced, 92.79% *gap*)
19	Staph. chromogenes	2,16	Staph. chromogenes
20	Staph. epidermidis	2,21	Staph. epidermidis
21	Staph. haemolyticus	1,78	Staph. haemolyticus
22	Staph. microti	2,04	Staph. simulans (sequenced, 99.89% *gap*)
24	Staph. petrasii	2,24	Staph. lettensis (sequenced, 99.29% *gap*)
25	Staph. haemolyticus	1,97	Staph. haemolyticus
26	Staph. sciuri	2,16	Staph. lyicus (sequenced, 98.69% *gap*)
27	Staph. pseudintermedius	1,96	Staph. pseudintermedius (sequenced, 98.81% *gap*)
28	Staph. simulans	2,35	Staph. simulans
29	Staph. haemolyticus	2,22	Staph. haemolyticus
32	No organism identification possible	1,44	Staph. devriesei (sequenced, 99.77% *gap*)
33	Staph. xylosus	1,88	Staph. devriesei
35	Staph. xylosus	1,81	Staph. pseudoxylosus (sequenced, 99.78% *gap*)
36	Staph. cohnii	1,7	Staph. haemolyticus
37	Staph. haemolyticus	2,2	Staph. haemolyticus
39	Staph. sciuri	2,23	Staph. chromogenes (sequenced, 98.75% *gap*)
40	Staph. epidermidis	2,21	Staph. epidermidis
41	Staph. epidermidis	2,22	Staph. epidermidis
42	Staph. caprae	2,13	Staph. caprae
43	Staph. chromogenes	2,42	Staph. chromogenes
44	Staph. arlettae	1,86	Staph. arlettae
45	Staph. xylosus	2,26	Staph. xylosus
46	Staph. capitis	2,21	Staph. capitis
47	Staph. simulans	2,41	Staph. simulans
48	Staph. epidermidis	2,18	Staph. epidermidis
49	No organism identification possible	1,6	Staph. haemolyticus (sequenced, 99.07% *gap*)
50	Staph. epidermidis	1,95	Staph. epidermidis
51	Staph. epidermidis	2,16	Staph. epidermidis
52	Staph. microti	2,05	Staph. muscae
53	Staph. warneri	1,98	Staph. warneri
54	Staph. equorum	2,31	Staph. equorum
56	Staph. warneri	2	Staph. warneri
57	Staph. chromogenes	2,42	Staph. chromogenes
59	Staph. haemolyticus	2,06	Staph. haemolyticus
60	Staph. haemolyticus	2,1	Staph. haemolyticus
62	Strep. uberis	2,28	Strep. uberis
63	Strep. uberis	2,18	Strep. uberis
64	Strep. uberis	2,41	Strep. uberis
65	Strep. uberis	2,29	Strep. uberis
66	Strep. uberis	2,06	Strep. uberis
67	Strep. uberis	1,99	Strep. uberis
68	Strep. uberis	2,2	Strep. uberis
69	Strep. uberis	2,28	Strep. uberis
70	Strep. uberis	2,31	Strep. uberis
71	Strep. uberis	2,3	Strep. uberis
72	Strep. uberis	2,32	Strep. uberis

73	Strep. uberis	2,22	Strep. uberis
74	Strep. uberis	2,21	Strep. uberis
75	Strep. uberis	2,22	Strep. uberis
76	Strep. uberis	2,28	Strep. uberis
77	Strep. uberis	2,15	Strep. uberis
78	Strep. uberis	2,26	Strep. uberis
79	Strep. uberis	2,3	Strep. uberis
80	Strep. uberis	1,8	Strep. uberis
81	Strep. uberis	2,26	Strep. uberis
82	Strep. uberis	1,87	Strep. uberis
83	Strep. uberis	2,4	Strep. uberis
84	Strep. uberis	2,22	Strep. uberis
85	Strep. uberis	2,38	Strep. uberis
86	Strep. uberis	2,2	Strep. uberis
87	Strep. uberis	2,36	Strep. uberis
88	Strep. uberis	2,34	Strep. uberis
90	Strep. uberis	2,43	Strep. uberis
92	Strep. uberis	2,26	Strep. uberis
93	Strep. uberis	2,21	Strep. uberis
94	Strep. uberis	2,15	Strep. uberis
95	Strep. dysgalactiae	2,22	Strep. dysgalactiae
96	Strep. uberis	2,33	Strep. uberis
98	Strep. uberis	2,3	Strep. uberis
99	Strep. uberis	2,37	Strep. uberis
100	Strep. uberis	2,31	Strep. uberis
101	Strep. uberis	2,13	Strep. uberis
102	Strep. uberis	2,26	Strep. uberis
104	Strep. uberis	2,39	Strep. uberis
105	Strep. uberis	2,17	Strep. uberis
106	Strep. uberis	2,35	Strep. uberis
107	Strep. uberis	2,43	Strep. uberis
108	Strep. uberis	2,29	Strep. uberis
109	Strep. uberis	2,15	Strep. uberis
110	Strep. uberis	2,13	Strep. uberis
112	Strep. uberis	2,35	Strep. uberis
113	Strep. uberis	2,3	Strep. uberis
114	Strep. uberis	2,28	Strep. uberis
115	Strep. uberis	2,3	Strep. uberis
116	Strep. uberis	2,08	Strep. uberis
117	Staph. chromogenes	1,93	Staph. chromogenes
118	Staph. chromogenes	2,35	Staph. chromogenes
120	Staph. chromogenes	2,41	Staph. chromogenes
121	Staph. simulans	2,19	Staph. simulans
122	Staph. simulans	2,29	Staph. simulans
123	Staph. haemolyticus	1,78	Staph. haemolyticus
124	Staph. epidermidis	2,09	Staph. epidermidis
125	Staph. epidermidis	2,09	Staph. epidermidis
126	Staph. epidermidis	2,07	Staph. epidermidis
127	Staph. epidermidis	2,11	Staph. epidermidis

128	Staph. chromogenes	2,25	Staph. chromogenes
129	Staph. epidermidis	2,07	Staph. epidermidis
130	Staph. chromogenes	2,19	Staph. chromogenes
131	Staph. chromogenes	2,22	Staph. chromogenes
132	Staph. epidermidis	1,98	Staph. epidermidis
133	Staph. chromogenes	2,31	Staph. chromogenes
134	Staph. chromogenes	2,39	Staph. chromogenes
135	Staph. chromogenes	2,22	Staph. chromogenes
136	Staph. epidermidis	2,08	Staph. epidermidis
137	Staph. epidermidis	2,09	Staph. epidermidis
138	Staph. epidermidis	2,16	Staph. epidermidis
139	Staph. chromogenes	2,23	Staph. chromogenes
140	Staph. chromogenes	2,27	Staph. chromogenes
142	Staph. epidermidis	2,18	Staph. epidermidis
143	Staph. warneri	2,04	Staph. warneri
144	Staph. haemolyticus	1,88	Staph. haemolyticus
146	Staph. epidermidis	2,23	Staph. epidermidis
148	Staph. equorum	2,06	Staph. equorum
149	Staph. petrasii	2,1	Staph. haemolyticus
150	Staph. epidermidis	2,12	Staph. epidermidis
151	Staph. chromogenes	2,17	Staph. chromogenes
152	Staph. chromogenes	2,35	Staph. chromogenes
153	Staph. chromogenes	2,25	Staph. chromogenes
154	Staph. simulans	2,29	Staph. simulans
155	Staph. chromogenes	2,22	Staph. chromogenes
156	Staph. chromogenes	2,2	Staph. chromogenes
157	Staph. haemolyticus	2,01	Staph. haemolyticus
158	Staph. chromogenes	2,2	Staph. chromogenes
159	Staph. haemolyticus	1,78	Staph. haemolyticus
160	Staph. epidermidis	2,09	Staph. epidermidis
161	Staph. chromogenes	2,19	Staph. chromogenes
162	Staph. epidermidis	2,21	Staph. epidermidis
163	Staph. simulans	2,3	Staph. simulans
164	Staph. chromogenes	2,33	Staph. chromogenes
165	Staph. simulans	2,19	Staph. simulans
166	Staph. chromogenes	2,14	Staph. chromogenes
167	Staph. epidermidis	2,17	Staph. epidermidis
168	Staph. epidermidis	2,03	Staph. epidermidis
169	Staph. chromogenes	2,11	Staph. chromogenes
170	Staph. chromogenes	2,21	Staph. chromogenes
171	Staph. epidermidis	2,29	Staph. epidermidis
172	Staph. epidermidis	2,03	Staph. epidermidis
173	Staph. chromogenes	2,44	Staph. chromogenes
174	Staph. epidermidis	2,09	Staph. epidermidis
175	Staph. chromogenes	2,34	Staph. chromogenes
176	Staph. chromogenes	2,22	Staph. chromogenes
177	Staph. epidermidis	2,32	Staph. epidermidis
178	Staph. epidermidis	2,22	Staph. epidermidis
179	Staph. epidermidis	2,19	Staph. epidermidis

180	Staph. haemolyticus	2,15	Staph. haemolyticus
181	Staph. chromogenes	2,03	Staph. chromogenes
183	Staph. microti	2,06	Staph. muscae
184	Staph. epidermidis	2,18	Staph. epidermidis
185	Staph. microti	1,91	Staph. muscae
186	Staph. equorum	2,2	Staph. equorum
187	Staph. caprae	2,13	Staph. caprae
188	Staph. haemolyticus	2,25	Staph. haemolyticus
189	Staph. caprae	2,11	Staph. caprae
190	Staph. simulans	2,25	Staph. simulans
191	Staph. chromogenes	2,28	Staph. chromogenes
192	Staph. chromogenes	2,26	Staph. chromogenes
193	Staph. epidermidis	2,21	Staph. epidermidis
194	Staph. epidermidis	2,15	Staph. epidermidis
195	Staph. caprae	1,97	Staph. caprae
196	Staph. chromogenes	1,85	Staph. chromogenes
197	Staph. haemolyticus	2,02	Staph. haemolyticus
198	Staph. epidermidis	2,11	Staph. epidermidis
199	Staph. chromogenes	2,42	Staph. chromogenes
200	Staph. chromogenes	2,33	Staph. chromogenes
201	Staph. haemolyticus	2,08	Staph. haemolyticus
202	Staph. haemolyticus	2,2	Staph. haemolyticus
203	Staph. chromogenes	2,3	Staph. chromogenes
204	Staph. chromogenes	2,16	Staph. chromogenes
205	Staph. caprae	2,07	Staph. caprae
206	Staph. chromogenes	2	Staph. chromogenes
207	Staph. haemolyticus	1,98	Staph. haemolyticus
208	Staph. haemolyticus	1,8	Staph. haemolyticus
209	Staph. chromogenes	2,21	Staph. chromogenes
210	Staph. caprae	2,02	Staph. caprae
211	Staph. haemolyticus	1,98	Staph. haemolyticus
212	Staph. epidermidis	2,01	Staph. epidermidis
213	Staph. chromogenes	2,12	Staph. chromogenes
214	Staph. petrasii	2,03	Staph. haemolyticus
215	Staph. haemolyticus	1,91	Staph. haemolyticus
216	Staph. epidermidis	2,11	Staph. epidermidis
217	Staph. haemolyticus	2,01	Staph. haemolyticus
218	Staph. caprae	1,92	Staph. caprae
219	Staph. chromogenes	2,27	Staph. chromogenes
220	Staph. chromogenes	2,1	Staph. chromogenes
222	Staph. haemolyticus	1,77	Staph. haemolyticus
223	Staph. epidermidis	2,11	Staph. epidermidis
224	Staph. epidermidis	2,15	Staph. epidermidis
225	Staph. caprae	1,83	Staph. caprae
226	Staph. caprae	2,06	Staph. caprae
227	Staph. chromogenes	1,99	Staph. chromogenes
228	Staph. simulans	2,26	Staph. simulans
229	Staph. chromogenes	2,39	Staph. chromogenes
230	Staph. epidermidis	2,2	Staph. epidermidis

231	Staph. chromogenes	2,34	Staph. chromogenes
232	Staph. lentus	1,8	Staph. epidermidis (sequenced, 98.48% *gap*)
233	Staph. haemolyticus	1,97	Staph. haemolyticus
234	Staph. epidermidis	1,88	Staph. epidermidis
235	Staph. simulans	2,37	Staph. simulans
236	Staph. chromogenes	2,26	Staph. chromogenes
237	Staph. epidermidis	2,23	Staph. epidermidis
238	Staph. epidermidis	1,97	Staph. epidermidis
239	Staph. simulans	2,21	Staph. simulans
240	Staph. epidermidis	2,21	Staph. epidermidis
241	Staph. epidermidis	2,02	Staph. epidermidis
242	Staph. epidermidis	2,22	Staph. epidermidis
243	Staph. caprae	2	Staph. caprae
244	Staph. chromogenes	1,86	Staph. chromogenes
245	Staph. caprae	2,01	Staph. caprae
246	Staph. simulans	2,36	Staph. simulans
247	Staph. epidermidis	2,16	Staph. epidermidis
248	Staph. chromogenes	2,23	Staph. chromogenes
249	Staph. succinus	1,93	Staph. epidermidis
250	Staph. chromogenes	2,25	Staph. chromogenes
251	Staph. epidermidis	2,08	Staph. epidermidis
252	Staph. haemolyticus	2	Staph. haemolyticus
253	Staph. epidermidis	2,16	Staph. epidermidis
254	Staph. microti	1,76	Staph. muscae
255	Staph. epidermidis	2,25	Staph. epidermidis
256	Staph. epidermidis	2,14	Staph. epidermidis
257	Staph. epidermidis	2,03	Staph. epidermidis
258	Staph. haemolyticus	2,04	Staph. haemolyticus
259	Staph. epidermidis	2,14	Staph. epidermidis
260	Staph. epidermidis	2,16	Staph. epidermidis
261	Staph. chromogenes	2,15	Staph. chromogenes
262	Staph. caprae	2,07	Staph. caprae
263	Staph. haemolyticus	1,92	Staph. haemolyticus
264	No organism identification possible	1,28	Staph. haemolyticus (sequenced, 98.26% *gap*)
265	Staph. chromogenes	2,19	Staph. chromogenes
266	Staph. epidermidis	2,15	Staph. epidermidis
267	Staph. haemolyticus	1,87	Staph. haemolyticus
268	Staph. chromogenes	2,29	Staph. chromogenes
269	Staph. chromogenes	2,2	Staph. chromogenes
270	Staph. chromogenes	2,18	Staph. chromogenes
271	Staph. simulans	2,31	Staph. simulans
272	Staph. epidermidis	2,17	Staph. epidermidis
274	Staph. caprae	2,22	Staph. caprae
276	Staph. chromogenes	2,25	Staph. chromogenes
277	Staph. epidermidis	2,07	Staph. epidermidis
278	Staph. epidermidis	2,1	Staph. epidermidis
279	Staph. epidermidis	2,01	Staph. epidermidis
280	Staph. epidermidis	2,16	Staph. epidermidis
281	Staph. haemolyticus	2,07	Staph. haemolyticus
282	Staph. chromogenes	2,24	Staph. chromogenes
283	Staph. xylosus	2,17	Staph. xylosus
284	Staph. chromogenes	2,21	Staph. chromogenes
285	Staph. epidermidis	2,07	Staph. epidermidis
286	Staph. chromogenes	2,31	Staph. chromogenes
287	Staph. epidermidis	2,12	Staph. epidermidis
288	Staph. haemolyticus	2	Staph. haemolyticus
289	Staph. haemolyticus	2,03	Staph. haemolyticus
290	Staph. haemolyticus	2,12	Staph. haemolyticus
291	Staph. haemolyticus	1,89	Staph. haemolyticus
292	Staph. haemolyticus	2,01	Staph. haemolyticus
294	Strep. uberis	2,16	Strep. uberis

Sheet 2: MALDI-TOF MS

Isolate N	MALDI-TOF MS	Log score	Species
2	Strep. gallolyticus	2,36	sheep
3	Strep. parauberis	2,17	sheep
4	Strep. dysgalactiae/Strep. canis	2,42	goat
7	Strep. suis	1,78	sheep
14	Strep. parauberis	2,18	sheep
15	Strep. uberis	2,48	sheep
18	Staph. microti	1,99	sheep
19	Staph. chromogenes	2,16	sheep
20	Staph. epidermidis	2,21	goat
21	Staph. haemolyticus	1,78	sheep
22	Staph. microti	2,04	sheep
24	Staph. petrasii	2,24	sheep
25	Staph. haemolyticus	1,97	sheep
26	Staph. sciuri	2,16	sheep
27	Staph. pseudintermedius	1,96	sheep
28	Staph. simulans	2,35	sheep
29	Staph. haemolyticus	2,22	sheep
32	No organism identification possible	1,44	sheep
33	Staph. xylosus	1,88	sheep
35	Staph. xylosus	1,81	sheep
36	Staph. cohni	1,7	sheep
37	Staph. haemolyticus	2,2	sheep
39	Staph. sciuri	2,23	sheep
40	Staph. epidermidis	2,21	sheep
41	Staph. epidermidis	2,22	sheep
42	Staph. caprae	2,13	sheep
43	Staph. chromogenes	2,42	sheep
44	Staph. arlettae	1,86	goat
45	Staph. xylosus	2,26	sheep
46	Staph. capitis	2,21	goat
47	Staph. simulans	2,41	goat
48	Staph. epidermidis	2,18	sheep
49	No organism identification possible	1,6	sheep
50	Staph. epidermidis	1,95	sheep
51	Staph. epidermidis	2,16	sheep
52	Staph. microti	2,05	sheep
53	Staph. warneri	1,98	sheep
54	Staph. equorum	2,31	sheep
56	Staph. warneri	2	sheep
57	Staph. chromogenes	2,42	sheep
59	Staph. haemolyticus	2,06	sheep
60	Staph. haemolyticus	2,1	sheep
62	Strep. uberis	2,28	sheep
63	Strep. uberis	2,18	sheep
64	Strep. uberis	2,41	sheep

65	Strep. uberis	2,29	sheep
66	Strep. uberis	2,06	sheep
67	Strep. uberis	1,99	sheep
68	Strep. uberis	2,2	sheep
69	Strep. uberis	2,28	sheep
70	Strep. uberis	2,31	sheep
71	Strep. uberis	2,3	sheep
72	Strep. uberis	2,32	sheep
73	Strep. uberis	2,22	sheep
74	Strep. uberis	2,21	sheep
75	Strep. uberis	2,22	sheep
76	Strep. uberis	2,28	sheep
77	Strep. uberis	2,15	sheep
78	Strep. uberis	2,26	sheep
79	Strep. uberis	2,3	sheep
80	Strep. uberis	1,8	sheep
81	Strep. uberis	2,26	sheep
82	Strep. uberis	1,87	sheep
83	Strep. uberis	2,4	sheep
84	Strep. uberis	2,22	sheep
85	Strep. uberis	2,38	sheep
86	Strep. uberis	2,2	sheep
87	Strep. uberis	2,36	sheep
88	Strep. uberis	2,34	sheep
90	Strep. uberis	2,43	sheep
92	Strep. uberis	2,26	sheep
93	Strep. uberis	2,21	sheep
94	Strep. uberis	2,15	sheep
95	Strep. dysgalactiae/Strep canis	2,22	sheep
96	Strep. uberis	2,33	sheep
98	Strep. uberis	2,3	sheep
99	Strep. uberis	2,37	goat
100	Strep. uberis	2,31	sheep
101	Strep. uberis	2,13	sheep
102	Strep. uberis	2,26	sheep
104	Strep. uberis	2,39	sheep
105	Strep. uberis	2,17	sheep
106	Strep. uberis	2,35	sheep
107	Strep. uberis	2,43	sheep
108	Strep. uberis	2,29	sheep
109	Strep. uberis	2,15	sheep
110	Strep. uberis	2,13	sheep
112	Strep. uberis	2,35	sheep
113	Strep. uberis	2,3	sheep
114	Strep. uberis	2,28	sheep
115	Strep. uberis	2,3	sheep
116	Strep. uberis	2,08	sheep
117	Staph. chromogenes	1,93	sheep
118	Staph. chromogenes	2,35	goat

120	Staph. chromogenes	2,41	sheep
121	Staph. simulans	2,19	sheep
122	Staph. simulans	2,29	sheep
123	Staph. haemolyticus	1,78	sheep
124	Staph. epidermidis	2,09	sheep
125	Staph. epidermidis	2,09	sheep
126	Staph. epidermidis	2,07	sheep
127	Staph. epidermidis	2,11	goat
128	Staph. chromogenes	2,25	goat
129	Staph. epidermidis	2,07	sheep
130	Staph. chromogenes	2,19	sheep
131	Staph. chromogenes	2,22	sheep
132	Staph. epidermidis	1,98	sheep
133	Staph. chromogenes	2,31	sheep
134	Staph. chromogenes	2,39	sheep
135	Staph. chromogenes	2,22	sheep
136	Staph. epidermidis	2,08	sheep
137	Staph. epidermidis	2,09	sheep
138	Staph. epidermidis	2,16	sheep
139	Staph. chromogenes	2,23	sheep
140	Staph. chromogenes	2,27	sheep
142	Staph. epidermidis	2,18	sheep
143	Staph. warneri	2,04	sheep
144	Staph. haemolyticus	1,88	sheep
146	Staph. epidermidis	2,23	sheep
148	Staph. equorum	2,06	sheep
149	Staph. petrasii	2,1	sheep
150	Staph. epidermidis	2,12	sheep
151	Staph. chromogenes	2,17	sheep
152	Staph. chromogenes	2,35	sheep
153	Staph. chromogenes	2,25	sheep
154	Staph. simulans	2,29	sheep
155	Staph. chromogenes	2,22	sheep
156	Staph. chromogenes	2,2	sheep
157	Staph. haemolyticus	2,01	sheep
158	Staph. chromogenes	2,2	sheep
159	Staph. haemolyticus	1,78	sheep
160	Staph. epidermidis	2,09	sheep
161	Staph. chromogenes	2,19	sheep
162	Staph. epidermidis	2,21	sheep
163	Staph. simulans	2,3	sheep
164	Staph. chromogenes	2,33	sheep
165	Staph. simulans	2,19	sheep
166	Staph. chromogenes	2,14	sheep
167	Staph. epidermidis	2,17	sheep
168	Staph. epidermidis	2,03	goat
169	Staph. chromogenes	2,11	sheep
170	Staph. chromogenes	2,21	sheep
171	Staph. epidermidis	2,29	goat

172	Staph. epidermidis	2,03	sheep
173	Staph. chromogenes	2,44	sheep
174	Staph. epidermidis	2,09	sheep
175	Staph. chromogenes	2,34	sheep
176	Staph. chromogenes	2,22	sheep
177	Staph. epidermidis	2,32	sheep
178	Staph. epidermidis	2,22	sheep
179	Staph. epidermidis	2,19	sheep
180	Staph. haemolyticus	2,15	sheep
181	Staph. chromogenes	2,03	sheep
183	Staph. microti	2,06	sheep
184	Staph. epidermidis	2,18	sheep
185	Staph. microti	1,91	sheep
186	Staph. equorum	2,2	sheep
187	Staph. caprae	2,13	sheep
188	Staph. haemolyticus	2,25	sheep
189	Staph. caprae	2,11	sheep
190	Staph. simulans	2,25	sheep
191	Staph. chromogenes	2,28	sheep
192	Staph. chromogenes	2,26	sheep
193	Staph. epidermidis	2,21	sheep
194	Staph. epidermidis	2,15	sheep
195	Staph. caprae	1,97	sheep
196	Staph. chromogenes	1,85	sheep
197	Staph. haemolyticus	2,02	sheep
198	Staph. epidermidis	2,11	sheep
199	Staph. chromogenes	2,42	sheep
200	Staph. chromogenes	2,33	sheep
201	Staph. haemolyticus	2,08	sheep
202	Staph. haemolyticus	2,2	sheep
203	Staph. chromogenes	2,3	sheep
204	Staph. chromogenes	2,16	sheep
205	Staph. caprae	2,07	sheep
206	Staph. chromogenes	2	sheep
207	Staph. haemolyticus	1,98	sheep
208	Staph. haemolyticus	1,8	sheep
209	Staph. chromogenes	2,21	sheep
210	Staph. caprae	2,02	sheep
211	Staph. haemolyticus	1,98	sheep
212	Staph. epidermidis	2,01	sheep
213	Staph. chromogenes	2,12	sheep
214	Staph. petrasii	2,03	sheep
215	Staph. haemolyticus	1,91	sheep
216	Staph. epidermidis	2,11	sheep
217	Staph. haemolyticus	2,01	sheep
218	Staph. caprae	1,92	sheep
219	Staph. chromogenes	2,27	sheep
220	Staph. chromogenes	2,1	sheep
222	Staph. haemolyticus	1,77	sheep

223	Staph. epidermidis	2,11	sheep
224	Staph. epidermidis	2,15	goat
225	Staph. caprae	1,83	sheep
226	Staph. caprae	2,06	sheep
227	Staph. chromogenes	1,99	sheep
228	Staph. simulans	2,26	sheep
229	Staph. chromogenes	2,39	sheep
230	Staph. epidermidis	2,2	sheep
231	Staph. chromogenes	2,34	sheep
232	Staph. lentus	1,8	goat
233	Staph. haemolyticus	1,97	sheep
234	Staph. epidermidis	1,88	sheep
235	Staph. simulans	2,37	sheep
236	Staph. chromogenes	2,26	sheep
237	Staph. epidermidis	2,23	sheep
238	Staph. epidermidis	1,97	sheep
239	Staph. simulans	2,21	sheep
240	Staph. epidermidis	2,21	goat
241	Staph. epidermidis	2,02	sheep
242	Staph. epidermidis	2,22	sheep
243	Staph. caprae	2	sheep
244	Staph. chromogenes	1,86	sheep
245	Staph. caprae	2,01	sheep
246	Staph. simulans	2,36	sheep
247	Staph. epidermidis	2,16	sheep
248	Staph. chromogenes	2,23	sheep
249	Staph. succinus	1,93	sheep
250	Staph. chromogenes	2,25	sheep
251	Staph. epidermidis	2,08	sheep
252	Staph. haemolyticus	2	sheep
253	Staph. epidermidis	2,16	sheep
254	Staph. microti	1,76	sheep
255	Staph. epidermidis	2,25	goat
256	Staph. epidermidis	2,14	sheep
257	Staph. epidermidis	2,03	sheep
258	Staph. haemolyticus	2,04	sheep
259	Staph. epidermidis	2,14	sheep
260	Staph. epidermidis	2,16	sheep
261	Staph. chromogenes	2,15	sheep
262	Staph. caprae	2,07	sheep
263	Staph. haemolyticus	1,92	sheep
264	No organism identification possible	1,28	sheep
265	Staph. chromogenes	2,19	sheep
266	Staph. epidermidis	2,15	sheep
267	Staph. haemolyticus	1,87	sheep
268	Staph. chromogenes	2,29	sheep
269	Staph. chromogenes	2,2	sheep
270	Staph. chromogenes	2,18	sheep
271	Staph. simulans	2,31	sheep

272	Staph. epidermidis	2,17	sheep
274	Staph. caprae	2,22	sheep
276	Staph. chromogenes	2,25	sheep
277	Staph. epidermidis	2,07	sheep
278	Staph. epidermidis	2,1	sheep
279	Staph. epidermidis	2,01	sheep
280	Staph. epidermidis	2,16	sheep
281	Staph. haemolyticus	2,07	sheep
282	Staph. chromogenes	2,24	sheep
283	Staph. xylosus	2,17	sheep
284	Staph. chromogenes	2,21	sheep
285	Staph. epidermidis	2,07	sheep
286	Staph. chromogenes	2,31	sheep
287	Staph. epidermidis	2,12	sheep
288	Staph. haemolyticus	2	sheep
289	Staph. haemolyticus	2,03	sheep
290	Staph. haemolyticus	2,12	sheep
291	Staph. haemolyticus	1,89	sheep
292	Staph. haemolyticus	2,01	sheep
294	Strep. uberis	2,16	sheep

MALDI-TOF MS result - Staph	N			MALDI-TOF MS result - Strep	N	
Staphylococcus epidermidis	59	28,9		Streptococcus uberis	51	89,5
Staphylococcus chromogenes	57	27,9		Streptococcus dysgalactiae/canis	2	3,5
Staphylococcus haemolyticus	32	15,7		Streptococcus parauberis	2	3,5
Staphylococcus caprae	13	6,4		Streptococcus gallolyticus	1	1,8
Staphylococcus simulans	13	6,4		Streptococcus suis	1	1,8
Staphylococcus microti	6	2,9		Unidentified	0	0,0
Staphylococcus xylosus	4	2,0		Total	57	100,0
Staphylococcus equorum	3	1,5				
Staphylococcus petrasii	3	1,5				
Staphylococcus warneri	3	1,5				
Staphylococcus sciuri	2	1,0				
Staphylococcus arlettae	1	0,5				
Staphylococcus capitis	1	0,5				
Staphylococcus cohni	1	0,5				
Staphylococcus lentus	1	0,5				
Staphylococcus psedintermedius	1	0,5				
Staphylococcus succinus	1	0,5				
Unidentified	3	1,5				
	204	100,0				

Sheet 3: PCR-RFLP by reference strains

Isolate N	PCR-RFLP	Species
2	Strep. gallolyticus	sheep
3	Unidentified strep	sheep
4	Strep. dysgalactiae	goat
7	Unidentified strep	sheep
14	Unidentified strep	sheep
15	Unidentified strep	sheep
18	Unidentified staph	sheep
19	Staph. chromogenes	sheep
20	Staph. epidermidis	goat
21	Staph. haemolyticus	sheep
22	Unidentified staph	sheep
24	Unidentified staph	sheep
25	Staph. haemolyticus	sheep
26	Unidentified staph	sheep
27	Unidentified staph	sheep
28	Staph. simulans	sheep
29	Staph. haemolyticus	sheep
32	Unidentified staph	sheep
33	Unidentified staph	sheep
35	Unidentified staph	sheep
36	Unidentified staph	sheep
37	Staph. haemolyticus	sheep
39	Unidentified staph	sheep
40	Staph. epidermidis	sheep
41	Staph. epidermidis	sheep
42	Staph. caprae	sheep
43	Staph. chromogenes	sheep
44	Staph. arlettae	goat
45	Staph. xylosus	sheep
46	Staph. capitis	goat
47	Staph. simulans	goat
48	Staph. epidermidis	sheep
49	Unidentified staph	sheep
50	Staph. epidermidis	sheep
51	Staph. epidermidis	sheep
52	Unidentified staph	sheep
53	Staph. warneri	sheep
54	Staph. equorum	sheep
56	Staph. warneri	sheep
57	Staph. chromogenes	sheep
59	Staph. haemolyticus	sheep
60	Staph. haemolyticus	sheep
62	Strep. uberis	sheep
63	Strep. uberis	sheep
64	Strep. uberis	sheep

65	Strep. uberis	sheep
66	Strep. uberis	sheep
67	Strep. uberis	sheep
68	Strep. uberis	sheep
69	Strep. uberis	sheep
70	Strep. uberis	sheep
71	Strep. uberis	sheep
72	Strep. uberis	sheep
73	Strep. uberis	sheep
74	Strep. uberis	sheep
75	Strep. uberis	sheep
76	Strep. uberis	sheep
77	Strep. uberis	sheep
78	Strep. uberis	sheep
79	Strep. uberis	sheep
80	Strep. uberis	sheep
81	Strep. uberis	sheep
82	Strep. uberis	sheep
83	Strep. uberis	sheep
84	Strep. uberis	sheep
85	Strep. uberis	sheep
86	Strep. uberis	sheep
87	Strep. uberis	sheep
88	Strep. uberis	sheep
90	Strep. uberis	sheep
92	Strep. uberis	sheep
93	Strep. uberis	sheep
94	Strep. uberis	sheep
95	Strep. dysgalactiae	sheep
96	Strep. uberis	sheep
98	Strep. uberis	sheep
99	Strep. uberis	goat
100	Strep. uberis	sheep
101	Strep. uberis	sheep
102	Strep. uberis	sheep
104	Strep. uberis	sheep
105	Strep. uberis	sheep
106	Strep. uberis	sheep
107	Strep. uberis	sheep
108	Strep. uberis	sheep
109	Strep. uberis	sheep
110	Strep. uberis	sheep
112	Strep. uberis	sheep
113	Strep. uberis	sheep
114	Strep. uberis	sheep
115	Strep. uberis	sheep
116	Strep. uberis	sheep
117	Staph. chromogenes	sheep
118	Staph. chromogenes	goat

120	Staph. chromogenes	sheep
121	Staph. simulans	sheep
122	Staph. simulans	sheep
123	Staph. haemolyticus	sheep
124	Staph. epidermidis	sheep
125	Staph. epidermidis	sheep
126	Staph. epidermidis	sheep
127	Staph. epidermidis	goat
128	Staph. chromogenes	goat
129	Staph. epidermidis	sheep
130	Staph. chromogenes	sheep
131	Staph. chromogenes	sheep
132	Staph. epidermidis	sheep
133	Staph. chromogenes	sheep
134	Staph. chromogenes	sheep
135	Staph. chromogenes	sheep
136	Staph. epidermidis	sheep
137	Staph. epidermidis	sheep
138	Staph. epidermidis	sheep
139	Staph. chromogenes	sheep
140	Staph. chromogenes	sheep
142	Staph. epidermidis	sheep
143	Staph. warneri	sheep
144	Staph. haemolyticus	sheep
146	Staph. epidermidis	sheep
148	Staph. equorum	sheep
149	Staph. haemolyticus	sheep
150	Staph. epidermidis	sheep
151	Staph. chromogenes	sheep
152	Staph. chromogenes	sheep
153	Staph. chromogenes	sheep
154	Staph. simulans	sheep
155	Staph. chromogenes	sheep
156	Staph. chromogenes	sheep
157	Staph. haemolyticus	sheep
158	Staph. chromogenes	sheep
159	Staph. haemolyticus	sheep
160	Staph. epidermidis	sheep
161	Staph. chromogenes	sheep
162	Staph. epidermidis	sheep
163	Staph. simulans	sheep
164	Staph. chromogenes	sheep
165	Staph. simulans	sheep
166	Staph. chromogenes	sheep
167	Staph. epidermidis	sheep
168	Staph. epidermidis	goat
169	Staph. chromogenes	sheep
170	Staph. chromogenes	sheep
171	Staph. epidermidis	goat

172	Staph. epidermidis	sheep
173	Staph. chromogenes	sheep
174	Staph. epidermidis	sheep
175	Staph. chromogenes	sheep
176	Staph. chromogenes	sheep
177	Staph. epidermidis	sheep
178	Staph. epidermidis	sheep
179	Staph. epidermidis	sheep
180	Staph. haemolyticus	sheep
181	Staph. chromogenes	sheep
183	Unidentified staph	sheep
184	Staph. epidermidis	sheep
185	Unidentified staph	sheep
186	Staph. equorum	sheep
187	Staph. caprae	sheep
188	Staph. haemolyticus	sheep
189	Staph. caprae	sheep
190	Staph. simulans	sheep
191	Staph. chromogenes	sheep
192	Staph. chromogenes	sheep
193	Staph. epidermidis	sheep
194	Staph. epidermidis	sheep
195	Staph. caprae	sheep
196	Staph. chromogenes	sheep
197	Staph. haemolyticus	sheep
198	Staph. epidermidis	sheep
199	Staph. chromogenes	sheep
200	Staph. chromogenes	sheep
201	Staph. haemolyticus	sheep
202	Staph. haemolyticus	sheep
203	Staph. chromogenes	sheep
204	Staph. chromogenes	sheep
205	Staph. caprae	sheep
206	Staph. chromogenes	sheep
207	Staph. haemolyticus	sheep
208	Staph. haemolyticus	sheep
209	Staph. chromogenes	sheep
210	Staph. caprae	sheep
211	Staph. haemolyticus	sheep
212	Staph. epidermidis	sheep
213	Staph. chromogenes	sheep
214	Staph. haemolyticus	sheep
215	Staph. haemolyticus	sheep
216	Staph. epidermidis	sheep
217	Staph. haemolyticus	sheep
218	Staph. caprae	sheep
219	Staph. chromogenes	sheep
220	Staph. chromogenes	sheep
222	Staph. haemolyticus	sheep

223	Staph. epidermidis	sheep
224	Staph. epidermidis	goat
225	Staph. caprae	sheep
226	Staph. caprae	sheep
227	Staph. chromogenes	sheep
228	Staph. simulans	sheep
229	Staph. chromogenes	sheep
230	Staph. epidermidis	sheep
231	Staph. chromogenes	sheep
232	Unidentified staph	goat
233	Staph. haemolyticus	sheep
234	Staph. epidermidis	sheep
235	Staph. simulans	sheep
236	Staph. chromogenes	sheep
237	Staph. epidermidis	sheep
238	Staph. epidermidis	sheep
239	Staph. simulans	sheep
240	Staph. epidermidis	goat
241	Staph. epidermidis	sheep
242	Staph. epidermidis	sheep
243	Staph. caprae	sheep
244	Staph. chromogenes	sheep
245	Staph. caprae	sheep
246	Staph. simulans	sheep
247	Staph. epidermidis	sheep
248	Staph. chromogenes	sheep
249	Staph. epidermidis	sheep
250	Staph. chromogenes	sheep
251	Staph. epidermidis	sheep
252	Staph. haemolyticus	sheep
253	Staph. epidermidis	sheep
254	Unidentified staph	sheep
255	Staph. epidermidis	goat
256	Staph. epidermidis	sheep
257	Staph. epidermidis	sheep
258	Staph. haemolyticus	sheep
259	Staph. epidermidis	sheep
260	Staph. epidermidis	sheep
261	Staph. chromogenes	sheep
262	Staph. caprae	sheep
263	Staph. haemolyticus	sheep
264	Unidentified staph	sheep
265	Staph. chromogenes	sheep
266	Staph. epidermidis	sheep
267	Staph. haemolyticus	sheep
268	Staph. chromogenes	sheep
269	Staph. chromogenes	sheep
270	Staph. chromogenes	sheep
271	Staph. simulans	sheep

272	Staph. epidermidis	sheep
274	Staph. caprae	sheep
276	Staph. chromogenes	sheep
277	Staph. epidermidis	sheep
278	Staph. epidermidis	sheep
279	Staph. epidermidis	sheep
280	Staph. epidermidis	sheep
281	Staph. haemolyticus	sheep
282	Staph. chromogenes	sheep
283	Staph. xylosus	sheep
284	Staph. chromogenes	sheep
285	Staph. epidermidis	sheep
286	Staph. chromogenes	sheep
287	Staph. epidermidis	sheep
288	Staph. haemolyticus	sheep
289	Staph. haemolyticus	sheep
290	Staph. haemolyticus	sheep
291	Staph. haemolyticus	sheep
292	Staph. haemolyticus	sheep
294	Strep. uberis	sheep

PCR-RFLP result - Staph	N		PCR-RFLP result - Strep	N
Unidentified Staphylococcus sp.	17		Unidentified	4
Staphylococcus arlettae	1		Streptococcus dysgalactiae	2
Staphylococcus capitis	1		Streptococcus gallolyticus	1
Staphylococcus caprae	13		Streptococcus uberis	50
Staphylococcus chromogenes	57		Total	57
Staphylococcus epidermidis	60			
Staphylococcus equorum	3			
Staphylococcus haemolyticus	34			
Staphylococcus simulans	13			
Staphylococcus warneri	3			
Staphylococcus xylosus	2			
	204			

Sheet 4: PCR-RFLP by amplicon sequencing

Isolate N	PCR-RFLP (gap gene sequencing identity)	Species
3	Strep. parauberis (sequenced, 99.2% *gap*)	sheep
7	Strep. ruminantium (sequenced, 97.42% *gap*)	sheep
14	Strep. parauberis (sequenced, 98.67% *gap*)	sheep
15	Strep. uberis (sequenced, 99.67% *gap*)	sheep
18	Staph. muscae (sequenced, 92.79% *gap*)	sheep
22	Staph. simulans (sequenced, 99.89% *gap*)	sheep
24	Staph. jettensis (sequenced, 99.29% *gap*)	sheep
26	Staph. hyicus (sequenced, 98.69% *gap*)	sheep
27	Staph. pseudintermedius (sequenced, 98.81% *gap*)	sheep
32	Staph. devriesei (sequenced, 99.77% *gap*)	sheep
33	Staph. devriesei (assigned by similarity to isolate 32)	sheep
35	Staph. pseudoxylosus (sequenced, 99.78% *gap*)	sheep
36	Staph. hemolyticus (sequenced, 98.91% *gap*)	sheep
39	Staph. chromogenes (sequenced, 98.75% *gap*)	sheep
49	Staph. haemolyticus (sequenced, 99.07% *gap*)	sheep
52	Staph. muscae (assigned by similarity to isolate 18)	sheep
183	Staph. muscae (assigned by similarity to isolate 18)	sheep
185	Staph. muscae (assigned by similarity to isolate 18)	sheep
232	Staph. epidermidis (sequenced, 98.48% *gap*)	goat
254	Staph. muscae (assigned by similarity to isolate 18)	sheep
264	Staph. haemolyticus (sequenced, 98.26% gap)	sheep

Sheet 5: Discordant IDs

Isolate N	MALDI-TOF MS	Log score	PCR-RFLP (gap gene sequencing identity)	Species
7	Strep. suis	1,78	Strep. ruminantium (sequenced, 97.42% *gap*)	sheep
18	Staph. microti	1,99	Staph. muscae (sequenced, 92.79% *gap*)	sheep
22	Staph. microti	2,04	Staph. simulans (sequenced, 99.89% *gap*)	sheep
24	Staph. petrasii	2,24	Staph. jettensis (sequenced, 99.29% *gap*)	sheep
26	Staph. sciuri	2,16	Staph. hyicus (sequenced, 98.69% *gap*)	sheep
32	No organism identification possible	1,44	Staph. devriesei (sequenced, 99.77% *gap*)	sheep
33	Staph. xylosus	1,88	Staph. devriesei (assigned by similarity to isolate 32)	sheep
35	Staphylococcus xylosus	1,81	Staph. pseudoxylosus (sequenced, 99.78% *gap*)	sheep
36	Staph. cohni	1,7	Staph. haemolyticus (sequenced, 98.91% *gap*)	sheep
39	Staph. sciuri	2,23	Staph. chromogenes (sequenced, 98.75% *gap*)	sheep
49	No organism identification possible	1,6	Staph. haemolyticus (sequenced, 99.07% *gap*)	sheep
52	Staph. microti	2,05	Staph. muscae (assigned by similarity to isolate 18)	sheep
149	Staph. petrasii	2,1	Staph. haemolyticus	sheep
183	Staph. microti	2,06	Staph. muscae (assigned by similarity to isolate 18)	sheep
185	Staph. microti	1,91	Staph. muscae (assigned by similarity to isolate 18)	sheep
214	Staph. petrasii	2,03	Staph. haemolyticus	sheep
232	Staphylococcus lentus	1,8	Staph. epidermidis (sequenced, 98.48% gap)	goat
249	Staph. succinus	1,93	Staph. epidermidis	sheep
254	Staph. microti	1,76	Staph. muscae (assigned by similarity to isolate 18)	sheep
264	No organism identification possible	1,28	Staph. haemolyticus (sequenced, 98.26% *gap*)	sheep

Additional file 6: Genomic sequence of the *gap* gene and sequence similarity data for staphylococci.

Isolate N. 18 (100979) - *Staphylococcus muscae* (Identity ID 92.79%)

GTAGAAATTGGTCGTTTAGCATTTAGAAGAAATTCAAGATGTAGAAAATATCGAAAGTTGTAGCAGTTAACGATTTAA
CAGACGATGACATGCTTGCACACTTATTAAAATATGACACAATGCAAGGACGTTTTACTGAAGAAGTAGAAGTAATTG
ACGGTGGGTTCCGCGTAAACGGTAAAGAAGTAAAATCATTCTCAGAACCAGAACCTTCAAAATTACCATGGGCTGACT
TAGGTGTAGATGTAGTGCTTGAATGTACAGGTTTCTTCACATCTAAAGAAAAAGCTGAAGCACACATTGAAGCAGGTG
CTAAAAAAGTATTAATCTCTGCTCCTGGTCAAGGCGACCTTAAAACAATCGTATACAATGTTAACCACGAATTATTAGA
CGGTTCTGAAACAGTAGTTTCTGGTGCATCATGTACTACAAACTCACTTGCACCAGTAGCTAAAACTTTACAAGATTCA
TTCGGTATCGTTGAAGGTCTTATGACTACAATTCACGCATACACTGGTGACCAAAATACACAAGACTCACCACACCGTA
AAGGCGACAAACGTCGTGCGCGTGCAGCTGCAGAAAACATCATTCCTAACTCAACAGGTGCTGCTAAAGCAATCGGT
CTTGTAATCCCAGAAATTGCTGGTAAATTAGACGGTGGCGCACAACGTGTACCAGTAGCAACAGGTTCATTAACTGAA
TTAACAGTAGTTCTTGAAAAAGAAGTAACAGTTGAAGAAGTTAACCAAGCAATGAAAGACGCAACTAACGAATCATTC
GGTTACACTGAAGACGAAATCGTATCTTCTGACGTAGTAGGTATGACATTCGGTGCATTATTCGATGCAACACA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus muscae glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1323	1323	100%	0.0	92.79%	931	DQ321694.1
☑	Staphylococcus muscae strain ATCC 49910 chromosome, complete genome	Staphylococcus ...	1301	1301	100%	0.0	92.36%	2095131	CP027848.1
☑	Staphylococcus muscae strain NCTC13833 genome assembly, chromosome: 1	Staphylococcus ...	1301	1301	100%	0.0	92.36%	2095116	LT906464.1
☑	Staphylococcus chromogenes glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1123	1123	100%	0.0	88.88%	931	AF495478.1
☑	Staphylococcus chromogenes strain 20B chromosome, complete genome	Staphylococcus ...	1118	1118	100%	0.0	88.77%	2424566	CP031471.1
☑	Staphylococcus chromogenes strain 1401 chromosome, complete genome	Staphylococcus ...	1112	1112	100%	0.0	88.67%	2350748	CP046028.1

Isolate N. 22 (82394) - *Staphylococcus simulans* (Identity ID 99.89%)

AGAATTGGTCGTTTAGCATTCAGAAGAATTCAAGATGTTGAAGGTATCGATGTAGTAGCAGTAAACGACTTAACAGAT
GATGAAATGTTAGCACACTTATTAAAATACGATACAATGCAAGGACGTTTCACTGAAGAAGTTGAAGTTGTAGATGGC
GGATTCCGCGTGAATGGTAAAGAAGTTAAATCATTCGAAGAACCAGATGCAAGCAAATTACCTTGGAAAGATTTAGA
CATTGATGTCGTATTAGAATGTACTGGTTTCTACACTAGCGACGAAAAAGCACAAGCTCACATTGACGCAGGTGCTAA
AAAAGTATTAATCTCTGCACCAGCAACTGGTGACGTTAAAACAATTGTTTATAACGTAAACCAAGATACTTTAGACAGC
TCTGACGTAATCGTTTCAGGTGCTTCTTGTACTACAAACTCACTTGCTCCAGTAGCAAAAGTATTAAATGACAGCTTCG
GTTTAGTAGAAGGTTTCATGACTACTATCCACGCTTACACTGGTGACCAAAATACTCAAGACGGTCCACACAGAAAAG
GCGACAAACGTCGTGCACGTGCAGCAGCTGAAAACATCGTTCCTAACTCAACTGGTGCTGCTAAAGCAATCGGTAAA
GTAATTCCTGAAATCGACGGAAAATTAGACGGTGGCGCTCAACGTGTTCCTGTAGCAACTGGTTCATTAACTGAATTA
ACAGTTGTATTAGACAAAGACGTAACAATCGAAGAAGTAAACGAAGCTATGAAAGCAGCTTCTAACGAATCATTCGG
TTACAACGAAGACGAAATCGTATCTTCAGACGTAGTTGGTATGACATTCGGTTCATTATTCGATGCAACTCAAACTCGT
GTTATGACTGTATCC

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus simulans strain MR1 chromosome, complete genome	Staphylococcus ...	1663	1663	100%	0.0	99.89%	2661512	CP015642.1
☑	Staphylococcus simulans strain MR2 chromosome, complete genome	Staphylococcus ...	1663	1663	100%	0.0	99.89%	2680372	CP016157.1
☑	Staphylococcus simulans strain MR4 chromosome, complete genome	Staphylococcus ...	1663	1663	100%	0.0	99.89%	2685102	CP017430.1
☑	Staphylococcus simulans strain MR3 chromosome, complete genome	Staphylococcus ...	1663	1663	100%	0.0	99.89%	2679685	CP017428.1
☑	Staphylococcus simulans glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1663	1663	100%	0.0	99.89%	931	DQ321698.1
☑	Staphylococcus simulans strain NCTC7944 genome assembly, chromosome: 1	Staphylococcus ...	1657	1657	100%	0.0	99.78%	2675237	LR134264.1

Isolate N. 24 (18886) - *Staphylococcus jettensis* (Identity ID 99.29%, Total score 1520)

GTATGATAACGCAAATGTACAGACTTAACTGATGACGAAATGTTAGCTCATTTATTAAAATATGACACTATGCAAGGTC
GCTTCACTAGTGAAGTTGAAGTTATCGATGGTGGTTTCCGTGTAAATGGTAAAGAAGTTAAATCTTACGAAGAACCAG
ATGCAAGCAAATTACCTTGGGGCGATTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACTGATAAAGAAA
AAGCAGAAGCTCACATCAATGCAGGAGCTAAAAAAGTATTAATTTCTGCACCAGCTAAAGGTGACGTTAAAACAATCG
TATTCAACACTAACCACAAAGACTTAGATGGTTCTGAAACAGTAGTATCAGGTGCTTCATGTACTACTAACTCATTAGC
ACCAGTTGCTAAAGTATTAAGCGATGAATTCGGTATCGTTGAAGGTTTAATGACAACTATCCACGCTTACACTGGTGA
CCAAATGACTCAAGACGGTCCTCACAAAAAAGGTGACAAACGTCGTGCTCGTGCAGCAGCTCAAAACATCGTACCTAA
CTCAACAGGTGCAGCTAAAGCTATCGGTAAAGTAATTCCTGAAATCGATGGTAAATTAGACGGTGGTGCACAACGTGT
ACCTGTAGCAACTGGTTCATTAACTGAAGTAACAGTTGTATTAGAAAAAGACGTTACAGTTGAAGACGTTAACAATGC
TATGAAAAACGCTTCAAACGAATCATTCGGTTACACTGAAGACGAAATCGTTTCTTCAGACGTAGTTGGTATGACTTAC
GGTTCATTATTCGATGCTACACAAAACTCGTGTAATGTCAGTTGGTGACCGTCAAATTTAGTTTAAAGTTTGCAGCTTG
GTATTGATAACGAAAAA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus jettensis strain SEQ258 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1520	1520	96%	0.0	99.29%	903	JN092108.1
☑	Staphylococcus jettensis strain SEQ257 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1506	1506	96%	0.0	98.93%	905	JN092107.1
☑	Staphylococcus jettensis strain SEQ256 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1506	1506	96%	0.0	98.93%	907	JN092106.1
☑	Staphylococcus jettensis strain SEQ255 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1506	1506	96%	0.0	98.93%	906	JN092105.1
☑	Staphylococcus jettensis strain SEQ110 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1504	1504	96%	0.0	98.93%	903	JN092110.1
☑	Staphylococcus jettensis strain SEQ259 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1504	1504	96%	0.0	98.93%	900	JN092109.1
☑	Staphylococcus petrasii subsp. petrasii strain CCM 8418 glyceraldehyde-3-phosphate dehydrogenase (gap) g...	Staphylococcus...	1502	1502	94%	0.0	99.40%	880	JX139895.1
☑	Staphylococcus jettensis strain SEQ036 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1498	1498	95%	0.0	98.93%	910	JN092104.1

Isolate N. 26 (62676) - *Staphylococcus hyicus* (Identity ID 98.69%)

GGTAGAATTGGTCGTTTAGCATTTAGAAGAATTCAAGACGTAGAAAAACATTGAGGTAGTAGGCTGTCAAATGATTTA
AACTGACGACGACATGCTTGCACACTTATTAAAAGTATGATACTATGCAAGGACGTTTTACTGAAGAAGTAGATGTAA
TTGATGGTGGTTTCCGCGTAAATGGTAAAGAAGTGAAATCATTCTCTGAACCAGAACCATCTAAATTACCTTGGAAAG
ATTTAGAAGTAGATGTTGTATTAGAATGTACTGGTTTCTTCACATCTAAAGAAAAAGCTGAAGCACACATCGAAGCTG
GTGCTAAAAAAGTTTTAATTTCAGCTCCAGGTACTGGCGATCTTAAAACAATCGTATACAACGTTAACCATGAAGAATT
AGACGGTTCAGAAACAGTTGTTTCAGGTGCATCTTGTACTACAAACTCATTAGCACCAGTAGCGAAAACATTACACGA
TGAATTTGGCATCGTTGAAGGTTTAATGACTACGATTCACGCTTACACAGGTGACCAAAATACACAAGATTCACCTCAC
AGAAAAGGTGACAAACGTCGTGCACGTGCAGCAGCTGAAAACATCATCCCTAACTCAACTGGTGCTGCAAAAGCAAT
CGGTTTAGTTATTCCAGAAATCGCTGGTAAATTAGATGGTGGGGCACAACGTGTACCAGTTGCTACAGGTTCATTAAC
AGAATTAACTGTAGTTTTAGAAAAAGAAGTTTCTGTTGAAGAAGTAAACAATGCTATGAAAAATGCAACGAATTATTG
TTTTGGTAGA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus hyicus strain UDI 129 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1626	1626	100%	0.0	98.69%	926	FJ578006.1
☑	Staphylococcus agnetis strain 1379 chromosome, complete genome	Staphylococcus...	1622	1622	99%	0.0	98.69%	2449249	CP045927.1
☑	Staphylococcus agnetis strain 12B chromosome, complete genome	Staphylococcus...	1622	1622	99%	0.0	98.69%	2345021	CP031266.1
☑	Staphylococcus agnetis strain 908 chromosome, complete genome	Staphylococcus...	1622	1622	99%	0.0	98.69%	2474434	CP009623.1
☑	Staphylococcus agnetis strain G4 chromosome	Staphylococcus...	1622	1622	99%	0.0	98.69%	2489066	CP081015.1
☑	Staphylococcus hyicus strain HY glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1520	1520	100%	0.0	96.63%	931	JQ728499.1
☑	Staphylococcus hyicus strain HDSY glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1482	1482	100%	0.0	95.87%	931	JQ728497.1

Isolate N. 27 (63653) - *Staphylococcus pseudintermedius* (Identity ID 98.81%)

GGTTTTTGGTGAGAAATTGGTCGTTTAGGCATTTAGAAGAATTCAAGATGTGGAAAAATATCGACGTTGTAGCAGTAA
AATGATTTAACAGACGACGATATGCTTGCACACTTATAAAAATATGACTCAACACAAGGTCGTTTTACTGAAGAAGTA
GAAGTAATTGACGGTGGTTTTCCGTGTAAATGGTAAAGAAGTGAAATCATTCTCTGAACCAGAAACCTAAAAACTTAC
CATGGGGCGAGTTAGACATCGACGTGGTATTAGAATGTACGGGTTTCTTCACTGATAAAGAAAAAGCTGAAGCACAC
ATCGAAGCAGGCGCGAAAAAAGTATTAATTTCTGCACCAGCTAAAGGTGACCTTAAAACAGTTGTATATAACGTTAAC
CACGAAATTTTAGATGGTACTGAAACAGTTGTTTCTGGTGCTTCATGTACAACAAACTCATTAGCACCTGTTGCCAAAA
CTTTACAAGACAACTTTGGTATCGTTGAAGGTTTAATGACAACAATTCACGCTTACACTGGTGACCAAAACACATTAGA
CGCACCTCACAGAAAAGGTGACAAACGTCGTGCGCGTGCAGCTGCTGAAAACATTATCCCTAACTCAACTGGTGCTGC
GAAAGCAATCGGTTTAGTTATTCCTGAAATCGATGGTAAATTAGACGGTGGTGCACAACGTGTTCCTGTAGCAACTGG
TTCATTAACTGAATTAACAGTTGTATTAGAAAAAGAAGTTTCAGTTGAAGAAGTTAACAAAGT

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus pseudintermedius strain MAD401 chromosome	Staphylococcus pseudintermedius	1639	1639	99%	0.0	98.81%	2883513	CP039742.1
☑	Staphylococcus pseudintermedius strain 063228 chromosome, complete genome	Staphylococcus pseudintermedius	1639	1639	99%	0.0	98.81%	2766566	CP015626.1
☑	Staphylococcus pseudintermedius strain ME4692 chromosome	Staphylococcus pseudintermedius	1633	1633	98%	0.0	98.91%	2750501	CP039747.1
☑	Staphylococcus pseudintermedius strain AH18 chromosome, complete genome	Staphylococcus pseudintermedius	1633	1633	98%	0.0	98.91%	2623199	CP030374.1
☑	Staphylococcus pseudintermedius strain 5912, complete genome	Staphylococcus pseudintermedius	1633	1633	99%	0.0	98.70%	2531995	CP009120.1
☑	Staphylococcus pseudintermedius strain 53_88 chromosome	Staphylococcus pseudintermedius	1633	1633	99%	0.0	98.70%	2593941	CP035740.1
☑	Staphylococcus pseudintermedius strain 53_60 chromosome	Staphylococcus pseudintermedius	1633	1633	98%	0.0	98.91%	2615959	CP035741.1
☑	Staphylococcus pseudintermedius strain 51_92 chromosome	Staphylococcus pseudintermedius	1633	1633	99%	0.0	98.70%	2512363	CP035742.1
☑	Staphylococcus pseudintermedius strain 49_44 chromosome	Staphylococcus pseudintermedius	1633	1633	98%	0.0	98.91%	2584763	CP035743.1

Isolate N. 32 (10255) - *Staphylococcus devriesei* (Identity ID 99.77%)

TTTTTGTAGAATTGGTCGTTTAGCATTTAGAAGAATTCAAGACGTAGAAGGTATTGAAGTAGTTGCAGTAAACGACTT
AACTGACGACGAAATGTTAGCTCATTTATTAAAATATGACACTATGCAAGGACGCTTCACTGGTGAAGTTGAAGTCGT
TGATGGTGGTTTCCGCGTAAATGGTAAAGAAGTTAAATCATACGAAGAACCAGACGCAAGCAAATTACCTTGGGGCG
ATTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACAGATAAAGAAAAAGCAGAAGCACACATTAATGCAG
GAGCTAAAAAAGTATTAATCTCTGCTCCAGCTAAAGGTGACGTTAAAACAATCGTATATAACACTAACCACAGCGACT
TAGACGGTTCAGAAACAGTTGTTTCAGGTGCTTCATGTACTACTAACTCATTAGCACCAGTTGCAAAAGTATTAAGTGA
TGAATTCGGTATCGTTGAAGGTTTAATGACTACTATCCATGCATACACTGGTGACCAAATGACTCAAGACGGTCCTCAT
AAAAAAGGCGACAAACGTCGTGCACGTGCAGCAGCTCAAAACATCGTACCTAACTCAACAGGTGCTGCTAAAGCTAT
CGGTAAAGTTATTCCTGAAATCGATGGTAAATTAGACGGCGGTGCACAACGTGTACCAGTAGCAACTGGTTCATTAAC
TGAAGTAACAGTTGTATTAGAAAAAGACGTTTCAGTTGAAGACGTTAACAATGCAATGAAAAATGCTTCAAACGAATC
ATTCGGTTACACTGAAGACGAAATCGTATCTTCAGACGTAGTAGGTATGACTTACGGTTCATTATTCGACGCTACACAA
ACTCGTGTAATGTCAGTAGGCGACCGTC

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus devriesei strain CCM 7896 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1615	1615	96%	0.0	99.77%	880	JX174278.1
☑	Staphylococcus devriesei strain F101 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1511	1511	90%	0.0	99.76%	824	KM251711.1
☑	Staphylococcus jettensis strain SEQ256 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1419	1419	99%	0.0	95.02%	907	JN092106.1
☑	Staphylococcus taiwanensis strain NTUH-S172 chromosome, complete genome	Staphylococcus ...	1417	1417	100%	0.0	94.73%	2517683	CP058667.1
☑	Staphylococcus jettensis strain SEQ110 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1417	1417	99%	0.0	95.01%	903	JN092110.1
☑	Staphylococcus jettensis strain SEQ257 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1417	1417	99%	0.0	95.01%	905	JN092107.1

Isolate N. 35 (22815) - *Staphylococcus pseudoxylosus* (Identity ID 99.78%)

GGTAGAATTGGTCGTTTAGCATTCAGAAGAATTCAAAACGTTGATGGAATTGACGTAGTAGCAGTAAATGATTTAACA
GATGACGAAATGTTAGCACATTTATTAAAATATGACACTATGCAAGGACGCTTCACAGGAGAAGTTGAAGTTGAAATG
ACGGTTTCCGTGTTAACGGACAAGAAGTAAAATCATTCTCTGAGCCAGAACCAAGTAAATTACCTTGGAAAGACTTAG
ACATTGATGTTGTATTAGAATGTACTGGTTTCTTCGCTGATAAAGAAAAAGCAGAAGCTCATATTGAAGCAGGTGCTA
AAAAAGTATTAATCTCTGCTCCAGCTACTGGTGACTTAAAAACAATCGTTTATAACACAAACCACCAAGAATTAGATGG
TTCAGAAACAGTAGTTTCAGGTGCTTCATGTACTACTAACTCATTAGCTCCAGTTGCAAAAGTATTAAACGATGACTTC
GGTTTAGTAGAAGGTTTCATGACTACAATCCATGCTTACACTGGTGACCAAAGCACACAAGATGCTCCACACAGAAAA
GGCGACAAACGTCGTGCGCGTGCAGCAGCTGAAAACATTATTCCTAACTCAACTGGTGCTGCTAAAGCAATTGGCCTA
GTAATCCCTGAAATCGATGGTAAATTAGACGGTGGAGCGCAACGTGTTCCTGTAGCTACTGGTTCTTTAACTGAATTA
ACAGTTGTATTAGAGAAAAATGTAAGTATTGAAGACGTTAATGCTTCAATGAAAAATGCATCAAACGAATCATTCGGT
TACACTGAAGATGAAATCGTTTCTTCTGACGTAATTGGTATGACTTACGGTTCATTATTCGATGCAACACAAACACGTG
TAA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus pseudoxylosus strain 14AME19 chromosome, complete genome	Staphylococcus...	1674	1674	99%	0.0	99.78%	2910290	CP068712.1
☑	Staphylococcus xylosus strain LZ glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1570	1570	93%	0.0	99.65%	860	JQ728502.1
☑	Staphylococcus xylosus strain SH-2 glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1563	1563	93%	0.0	99.53%	859	JQ728507.1
☑	Staphylococcus xylosus strain JM glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1522	1522	93%	0.0	98.94%	862	JQ728501.1
☑	Staphylococcus xylosus partial gap gene for glyceraldehyde-3-phosphate dehydrogenase, strain C8	Staphylococcus...	1493	1493	89%	0.0	99.75%	815	LK023497.1

Isolate N. 36 (6441) - *Staphylococcus haemolyticus* (Identity ID 98.91%)

TTTTTGGTAGAAATTGGTCGTTTAGCATTTAGAAGAAATTCAAGACGTAGAAGGTATTGAAGTAGTTGCAGTAAACGA
CTTAACAGACGACGAAATGTTAGCTCATTTATTAAAATATGACACTATGCAAGGTCGTTTTACAGGAGAAGTTGAAGT
TATTGATGGTGGTTTCCGCGTAAATGGTAAAGAAGTTAAATCATACGAAGAACCAGATGCAAGCAAATTACCTTGGG
GCGATTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACAGATAAAGAAAAAGCTGAAGCACACATCAATG
CAGGTGCTAAAAAAGTATTAATCTCTGCACCAGCTAAAGGTGATGTTAAAACAATCGTATTCAACACTAACCACAATG
ACTTAGATGGTTCAGAAACAGTTGTTTCAGGTGCATCATGTACTACTAACTCATTAGCACCAGTTGCTAAAGTGTTAAG
TGACGAATTTGGTTTAGTTGAAGGTTTAATGACAACTATTCACGCATACACTGGTGACCAAATGACTCAAGACGGTCC
ACATAAAAAAGGCGATAAACGTCGTGCGCGTGCAGCAGCTCAAAACATCGTACCAAACTCAACAGGTGCTGCAAAAG
CTATCGGTAAAGTTATTCCTGAAATCGATGGTAAATTAGACGGTGGTGCTCAACGTGTACCAGTTGCTACAGGTTCATT
AACTGAAGTAACAGTTGTATTAGAAAAAGACGTTACTGTTGAAGACGTTAACAAAGCAATGAAAAACGCTTCAAACG
AATCATTCGGTTACACTGAAGACGAAATCGTTTCTTCAGACGTAGTTGGTATGACTTACGGTTCATTATTCGATGCTAC
TCAAACTCGTGTAATG

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus haemolyticus strain GDY8P80P chromosome, complete genome	Staphylococcus...	1639	1639	99%	0.0	98.91%	2715577	CP063443.1
☑	Staphylococcus haemolyticus strain SE2.14 chromosome, complete genome	Staphylococcus...	1600	1600	99%	0.0	98.15%	2323230	CP084235.1
☑	Staphylococcus haemolyticus strain SE3.8 chromosome, complete genome	Staphylococcus...	1600	1600	99%	0.0	98.15%	2323407	CP084229.1
☑	Staphylococcus haemolyticus strain NY5 chromosome, complete genome	Staphylococcus...	1600	1600	99%	0.0	98.15%	2472146	CP078159.1
☑	Staphylococcus haemolyticus strain SE3.9 chromosome, complete genome	Staphylococcus...	1600	1600	99%	0.0	98.15%	2323296	CP049091.1
☑	Staphylococcus haemolyticus strain SCAID URN1-2019 chromosome	Staphylococcus...	1589	1589	99%	0.0	97.93%	2256190	CP052055.1
☑	Staphylococcus haemolyticus isolate Staphylococcus haemolyticus K8 genome assembly, chromosome: I	Staphylococcus...	1589	1589	99%	0.0	97.93%	2426012	LT963441.1
☑	Staphylococcus haemolyticus strain 12b chromosome, complete genome	Staphylococcus...	1589	1589	99%	0.0	97.93%	2339810	CP071505.1
☑	Staphylococcus haemolyticus strain 7b chromosome, complete genome	Staphylococcus...	1589	1589	99%	0.0	97.93%	2341142	CP071508.1
☑	Staphylococcus haemolyticus strain 1b chromosome, complete genome	Staphylococcus...	1589	1589	99%	0.0	97.93%	2339731	CP071512.1
☑	Staphylococcus haemolyticus JCSC1435 DNA, complete genome	Staphylococcus...	1589	1589	99%	0.0	97.93%	2685015	AP006716.1
☑	Staphylococcus haemolyticus strain VB5326 chromosome, complete genome	Staphylococcus...	1583	1583	99%	0.0	97.83%	2699292	CP045137.2
☑	Staphylococcus haemolyticus strain VB19458 chromosome, complete genome	Staphylococcus...	1583	1583	99%	0.0	97.83%	2699210	CP045187.2

Isolate N. 39 (22) - *Staphylococcus chromogenes* (Identity ID 98.75%)

CGTGTTTGAGTTGCATCAAATAATGCACCAAAAGTCATGCCTACAACATCTGAAGATACGATTTCGTCTTCAGTGTAAC
CGAATGATTCGTTAGTTGCATTTTTCATTGCATTGTTAACGTCTTCTACTGATACTTCTTTATCTAAAACAACTGTTAATT
CAGTTAATGAACCTGTTGCTACTGGTACACGTTGTGCGCCACCGTCTAATTTTCCATCAATTTCAGGGATAACTAAACC
GATTGCTTTCGCAGCACCTGTTGAGTTAGGAATAATGTTTTCTGCAGCTGCACGTGCACGACGTTTGTCACCTTTTCTG
TGTGGTGAGTCTTGTGTATTTTGGTCACCAGTGTATGCGTGAATTGTAGTCATTAAACCTTCAACGATACCAAATTCAT
CGTTTAAAGTTTTTGCTACTGGTGCTAATGAGTTTGTTGTACAAGAAGCACCTGATACAACTGTTTCAGAACCGTCTAA
TTCTTCATGGTTGACATTATATACGATTGTTTTAAGATCGCCAGTTCCTGGTGCAGAAATTAATACTTTTTTAGCACCTG
CTTCAATGTGAGTTTCTGCTTTTTCTTTTGATGTAAAGAAACCTGTACATTCTAAAACAACATCTACGTCAAGATCTTTCC
ATGGTAATTTTGATGGTTCTGGTTCAGAGAATGATTTCACTTCTTTACCATTTACGCGGAAACCACCATCAATTACATCT
ACTTCTTCAGTAAAACGACCTTGCATTGTGTCATATTTCAATAAATGTGCAAGCATATCGTCGTCTGTTAAATCGTTTAC
AGCTACAACCTCAATATTTTCTACGTCTTGAATTCTTCTGAATGCTAAACGAC

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus chromogenes strain 20B chromosome, complete genome	Staphylococcus...	1561	1561	100%	0.0	98.75%	2424566	CP031471.1
☑	Staphylococcus chromogenes glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1561	1561	100%	0.0	98.75%	931	AF495478.1
☑	Staphylococcus chromogenes strain 1401 chromosome, complete genome	Staphylococcus...	1555	1555	100%	0.0	98.64%	2350748	CP046028.1
☑	Staphylococcus chromogenes strain 17A chromosome, complete genome	Staphylococcus...	1555	1555	100%	0.0	98.64%	2351540	CP031274.1
☑	Staphylococcus chromogenes strain 34B chromosome, complete genome	Staphylococcus...	1550	1550	100%	0.0	98.52%	2369172	CP031470.1
☑	Staphylococcus chromogenes strain SG glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus...	1548	1548	99%	0.0	98.63%	892	JQ728506.1

Isolate N. 49 (17963) - *Staphylococcus haemolyticus* (Identity ID 99.07%)

GGTAGAATTGGTCGTTTAGCATTTAGAAGAAATTCAAAGACGTAGAAAGGTATTGAAGTAGTTGCAGTAAACGACTT
AACAGACGACGAAATGTTAGCTCATTTATTAAAATATGACACTATGCAAGGTCGTTTTACAGGAGAAGTTGAAGTTAT
TGATGGTGGTTTCCGCGTAAATGGTAAAGAAGTTAAATCATACGAAGAACCAGATGCAAGCAAATTACCTTGGGGCG
ATTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACAGATAAAGAAAAAGCTGAAGCACACATCAATGCAG
GTGCTAAAAAAGTATTAATCTCTGCACCAGCTAAAGGTGATGTTAAAACAATCGTATTCAACACTAACCACAATGACTT
AGATGGTTCAGAAACAGTTGTTTCAGGTGCATCATGTACTACTAACTCATTAGCACCAGTTGCTAAAGTGTTAAGTGAC
GAATTTGGTTTAGTTGAAGGTTTAATGACAACTATTCACGCATACACTGGTGACCAAATGACTCAAGACGGTCCACAT
AAAAAAGGCGATAAACGTCGTGCGCGTGCAGCAGCTCAAAACATCGTACCAAACTCAACAGGTGCTGCAAAAGCTAT
CGGTAAAGTTATTCCTGAAATCGATGGTAAATTAGACGGTGGTGCTCAACGTGTACCAGTTGCTACAGGTTCATTAAC
TGAAGTAACAGTTGTATTAGAAAAAGACGTTACTGTTGAAGACGTTAACAAAGCAATGAAAAACGCTTCAAACGAATC
ATTCGGTTACACTGAAGACGAAATCGTTTCTTCAGACGTAGTTGGTATGACTTACGGTTCATTATTCGATGCTACTCAA
A

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus haemolyticus strain GDY8P80P chromosome, complete genome	Staphylococcus...	1574	1574	100%	0.0	98.98%	2715577	CP063443.1
☑	Staphylococcus haemolyticus partial gap gene for glyceraldehyde-3-phosphate dehydrogenase, strain C4	Staphylococcus...	1539	1539	97%	0.0	99.07%	864	LK023494.1
☑	Staphylococcus haemolyticus strain SE2.14 chromosome, complete genome	Staphylococcus...	1535	1535	100%	0.0	98.18%	2323230	CP084235.1
☑	Staphylococcus haemolyticus strain SE3.8 chromosome, complete genome	Staphylococcus...	1535	1535	100%	0.0	98.18%	2323407	CP084229.1
☑	Staphylococcus haemolyticus strain NY5 chromosome, complete genome	Staphylococcus...	1535	1535	100%	0.0	98.18%	2472146	CP078159.1
☑	Staphylococcus haemolyticus strain SE3.9 chromosome, complete genome	Staphylococcus...	1535	1535	100%	0.0	98.18%	2323296	CP049091.1

Isolate N. 232 (10511) - *Staphylococcus epidermidis* (Identity ID 98.48%)

GGTAGAATTTGGTCGTTTAGCATTTCAGAAAGAATTCAAGATGTAGAAGGTCTTGAAAGTAGTTGCAGTTAAACGACT
TAACAGATGACGATATGTTAGCTCATTTATTAAAATACGATACTATGCAAGGTCGTTTCACTGGAGAAGTTGAAGTTAT
CGAAGGTGGATTCCGTGTTAACGGTAAAGAAATTAAATCATTCGATGAACCAGATGCTGGTAAATTACCATGGGGCG
ATTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACTGATAAAGAAAAAGCACAAGCTCACATCGATGCAG
GTGCTAAAAAAGTATTAATCTCAGCTCCAGCTAAAGGTGATGTAAAAACAATCGTATTCAACACTAACCATGACACATT
AGATGGTTCAGAAACAGTTGTTTCAGGTGCTTCTTGTACTACTAACTCATTAGCACCAGTTGCAAAAGTTTTAAGTGAT
GAATTCGGTTTAGTTGAAGGTTTCATGACTACAATTCACGCTTACACTGGTGACCAAAATACACAAGACGCACCTCACA
GAAAAGGTGACAAACGTCGTGCACGTGCAGCAGCAGAAAATATTATCCCTAACTCAACAGGTGCTGCTAAAGCTATC
GGTAAAGTTATTCCAGAAATCGATGGTAAATTAGACGGTGGAGCACAACGTGTTCCAGTTGCTACTGGTTCTTTAACT
GAATTAACTGTAGTATTAGACAAACAAGATGTAACTGTTGACCAAGTTAACAGTGCTATGAAACAAGCTTCAGACGAA
TCATTCGGTTACACTGAAGACGAAATCGTATCTTCTGATATCGTTGGTATGACTTACGGTTCATTATTCGATGCGACTC
AAACTCGTGTTAGACTA

	Description	Scientific Name	Common Name	Taxid	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☐	Staphylococcus epidermidis strain SESURV_p2_0614 chromosome	Staphyl...	NA	1282	1646	1646	99%	0.0	99.23%	2523089	CP043788.1
☐	Staphylococcus epidermidis strain TMDU-128 chromosome, complete genome	Staphyl...	NA	1282	1646	1646	99%	0.0	99.23%	2551919	CP093170.1
☐	Staphylococcus epidermidis strain CBPA-ST-11003 chromosome, complete genome	Staphyl...	NA	1282	1646	1646	99%	0.0	99.23%	2566999	CP071992.1
☐	Staphylococcus epidermidis strain HD104-2 chromosome, complete genome	Staphyl...	NA	1282	1646	1646	99%	0.0	99.23%	2453988	CP053007.1
☐	Staphylococcus epidermidis strain 14.1.R1 chromosome, complete genome	Staphyl...	NA	1282	1618	1618	99%	0.0	98.69%	2572575	CP018842.1
☐	Staphylococcus epidermidis strain BYQ glyceraldehyde-3-phosphate dehydrogenase (gap) gene, par...	Staphyl...	NA	1282	1616	1616	100%	0.0	98.48%	927	JQ728491.1
☐	Staphylococcus epidermidis strain sep1 chromosome	Staphyl...	NA	1282	1613	1613	99%	0.0	98.58%	2536282	CP101316.1

Isolate N. 264 (85364) - *Staphylococcus haemolyticus* (Identity ID 98.26%)

TTTTGGTAGAATTGGTCGTTTAGCATTTAGAAGAATTCAAGACGTAGAAGGTATTGAAGTAGTGCAGTAAACGACTTA
ACAGACGACGAAATGTTAGCTCATTTATTAAAATATGACACTATGCAAGGTCGTTTCACAGGAGAAGTTGAAGTTATT
GATGGTGGTTTCCGCGTAAATGGTAAAGAAGTTAAATCATACGAAGAACCAGATGCAAGCAAATTACCTTGGGGCGA
TTTAGATATCGACGTAGTATTAGAATGTACTGGTTTCTATACAGATAAAGAAAAAGCAGAAGCACACATCAATGCAGG
TGCTAAAAAAGTATTAATCTCTGCACCAGCTAAAGGTGATGTTAAAACAATCGTATTCAACACTAACCACAATGACTTA
GATGGTTCAGAAACAGTTGTTTCAGGTGCATCATGTACTACTAACTCATTAGCACCAGTTGCTAAAGTGTTAAGTGAC
GAATTTGGTTTAGTTGAAGGTTTAATGACAACTATTCACGCATACACTGGTGACCAAATGACTCAAGACGGTCCACAT
AAAAAAGGCGACAAACGTCGTGCGCGTGCAGCAGCTCAAAACATCGTACCAAACTCAACAGGTGCTGCAAAAGCTAT
CGGTAAAGTTATTCCTGAAATCGATGGTAAATTAGACGGTGGTGCTCAACGTGTACCAGTTGCTACAGGTTCATTAAC
TGAAGTAACAGTTGTATTAGAAAAAGACGTTACTGTTGAAGACGTTAACAAAGCAATGAAAAACGCTTCAAACGAATC
ATTCGGTTACACTGAAGACGAAATCGTTTGCTTCAGACGTAGTATGAGTATGACTTACGGTTCATTATTCGAGGCTACT

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Staphylococcus haemolyticus strain GDY8P80P chromosome, complete genome	Staphylococcus ...	1659	1659	99%	0.0	99.35%	2715577	CP063443.1
☑	Staphylococcus haemolyticus strain SE2.14 chromosome, complete genome	Staphylococcus ...	1620	1620	99%	0.0	98.58%	2323230	CP084235.1
☑	Staphylococcus haemolyticus strain SE3.8 chromosome, complete genome	Staphylococcus ...	1620	1620	99%	0.0	98.58%	2323407	CP084229.1
☑	Staphylococcus haemolyticus strain NY5 chromosome, complete genome	Staphylococcus ...	1620	1620	99%	0.0	98.58%	2472146	CP078159.1
☑	Staphylococcus haemolyticus strain SE3.9 chromosome, complete genome	Staphylococcus ...	1620	1620	99%	0.0	98.58%	2323296	CP049091.1
☑	Staphylococcus haemolyticus strain SCAID URN1-2019 chromosome	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2256190	CP052055.1
☑	Staphylococcus haemolyticus isolate Staphylococcus haemolyticus K8 genome assembly, chromosome: I	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2426012	LT963441.1
☑	Staphylococcus haemolyticus strain 12b chromosome, complete genome	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2339810	CP071505.1
☑	Staphylococcus haemolyticus strain 7b chromosome, complete genome	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2341142	CP071508.1
☑	Staphylococcus haemolyticus strain 1b chromosome, complete genome	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2339731	CP071512.1
☑	Staphylococcus haemolyticus JCSC1435 DNA, complete genome	Staphylococcus ...	1609	1609	99%	0.0	98.37%	2685015	AP006716.1
☑	Staphylococcus haemolyticus glyceraldehyde-3-phosphate dehydrogenase (gap) gene, partial cds	Staphylococcus ...	1607	1607	99%	0.0	98.26%	931	DQ321687.1
☑	Staphylococcus haemolyticus strain VB5326 chromosome, complete genome	Staphylococcus ...	1604	1604	99%	0.0	98.26%	2699292	CP045137.2

Additional file 7: Genomic sequence of the gap gene and sequence similarity data for streptococci.

Isolate N. 3 (15004) - *Streptococcus parauberis* (Identity ID 99.20%)

TAACTAATTGATTTCCATCAACAGTTTTGTACTTTAGTTTGAGTAGCATCGAATAATGAACCGAAAGACATACCAACGA
TATCAGATGATACGATTGGATCTTCAGTGTAACCATATGAATCATTAGCTGCAGCTTTCATTACTGAGTTAATTTCTTCT
ACTGAAGTTTCTTTATTAAGAACTGCTACTAATTCTGTTACTGAACCTGTTGGAACTGGTACACGTTGTGCAGCACCGT
CAAGTTTACCATTTAATTCAGGGATAACAAGACCGATTGCTTTAGCAGCACCAGTTGAGTTAGGAACGATATTGTTAG
CACCAGCACGGGCACGACGTAAGTCACCACCACGGTGAGGTCCATCAAGAAGCATTTGATCCCCAGTGTAAGCGTGG
ATTGTAGTCATTAAACCTTGTTTTACGCCAAAGTTATCTTGTAAAGCTTTAGCCATTGGAGCTAAACAGTTTGTAGTACA
TGAAGCACCTGAAATAACTGTTTCAGTTCCATCAAGGATATCATGGTTAGTGTTAAATACAACTGTTTTCACGTCATCTC
CACCAGGAGCAGTGATAACAACTTTTTTAGCACCATTTTCATGTAAATGTTTTTCAGCAGCTGCTTTTTTAGCAAAGAA
ACCAGTTGCTTCAAGAACGATTTCAACACCGTCAGTTGCCCAGTCAATTTGTTCTGGATCTTTTTCAGCAGAAACTTTAA
TGAATTTTCCGTTAACGTCAAATCCACCATCTTTAACTTCTACAGTACCGTCAAAACGACCTTGAGTTTGTATCGTATTTT
AACAAGTGTGCAAGCATATTTGGGATCTGTAAAGGTCGTTGATGCGAGTAACTTCAACACCTTCCTACATTTTGAATAC
GACGG

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Streptococcus parauberis strain SPOF3K chromosome, complete genome	Streptococcus p...	1581	1581	100%	0.0	99.32%	2128740	CP025420.1
☑	Streptococcus parauberis KCTC 11537, complete genome	Streptococcus p...	1576	1576	100%	0.0	99.20%	2143887	CP002471.1
☑	Streptococcus parauberis glyceraldehyde-3-phosphate dehydrogenase gene, complete cds	Streptococcus p...	1576	1576	100%	0.0	99.20%	1011	AF421901.1
☑	Streptococcus uberis strain SS131025-3 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete ...	Streptococcus u...	1266	1266	100%	0.0	92.82%	1011	KU588171.1
☑	Streptococcus uberis strain SS131025-2 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete ...	Streptococcus u...	1266	1266	100%	0.0	92.82%	1011	KU588170.1
☑	Streptococcus uberis strain SS131025-1 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete ...	Streptococcus u...	1266	1266	100%	0.0	92.82%	1011	KU588169.1
☑	Streptococcus uberis 0140J complete genome	Streptococcus u...	1149	1149	100%	0.0	90.42%	1852352	AM946015.1

Isolate N. 7 (42465) - *Streptococcus ruminantium* (ID 97.42%)

TTTCGATTGTCGTACCCATGATACAACTTTCACCAATTGCTCGCCCATCCACTTCAATAACTTTTAGTTTGAGATGCGTC
AAACAATGAACAGAAATGAGATACCCACGATGTCTGAAGATACAAAGTGGATCTTCAGTGTAACCGAATGATTCAGT
AGCAGCAGCTTTCATAGCAGCGTTTACTTCTTCAGCAGTTACTTTCTTATCAAGAGTCGCAACCAATTCAGTTACAGAA
CCTGTTGGAAACTGGAACACGTTGTGCGGCACCATCAAGTTTACCATTCAATTCTGGGATTACCAAACCGATAGCTTTA
GCTGCACCAGTTGAGTTAGGAACGATGTTTGCTGCAGCAGCACGAGCACGACGAAGGTCACCACCACGGTGTGGTCC
GTCAAGAACCATTTGGTCACCAGTGTAACCGTGGATTGTAGTCATCAAACCTTTTTGAACGCCAAACGCATCGTGAAG
AGCTTTAGCCATTGGTGCCAAACAGTTTGTAGTACATGAAGCACCTGAGATAACTGTTTCAGTACCGTCAAGGATGTC
ATGGTTAGTGTTGAAAACAACTGTCTTCACATCGTTACCACCAGGAGCAGTGATAACAACTTTCTTAGCACCGTTAGCG
TGAATGTGTTGCTCAGCTTTTTCTTTAGAAGCAAAGAAACCTGTTGCTTCCAAAACAATATCTACGCCATCAGTAGCCC
AGTCAATGTTTCCTGGCTCACGCTCAGCAGAAACTTTAAACAGAATTTACCCGTTAACTTCAAAAACCACCCGTCTTTAA
CTTACAACCAGTACCCATCGAAAACGACCCTTTGA

			Score	Score	Cover	E value	Ident		
☑	Streptococcus sp. DAT741 chromosome, complete genome	Streptococcus sp. DAT741	1375	1375	99%	0.0	97.42%	2105284	CP019557.1
☑	Streptococcus ruminantium GUT-189 DNA, complete genome	Streptococcus ruminantium	1375	1375	99%	0.0	97.42%	2081190	AP025333.1
☑	Streptococcus ruminantium GUT-183 DNA, complete genome	Streptococcus ruminantium	1375	1375	99%	0.0	97.42%	2175328	AP025331.1
☑	Streptococcus ruminantium GUT187T DNA, complete genome	Streptococcus ruminantium	1369	1369	99%	0.0	97.30%	2090539	AP018400.1
☑	Streptococcus ruminantium GUT-184 DNA, complete genome	Streptococcus ruminantium	1369	1369	99%	0.0	97.30%	2115310	AP025332.1
☑	Streptococcus suis strain NCTC10237 genome assembly, chromosome: 1	Streptococcus suis	1214	1214	99%	0.0	93.87%	2070644	LR594043.1
☑	Streptococcus suis strain 1081 chromosome, complete genome	Streptococcus suis	1214	1214	99%	0.0	93.87%	2228089	CP017667.1
☑	Streptococcus suis strain 0061 chromosome, complete genome	Streptococcus suis	1214	1214	99%	0.0	93.87%	2138420	CP017666.1

Isolate N. 14 (86773) - *Streptococcus parauberis* (Identity ID 98.67%)

CCTNCTCCAAGTGTCGAACAAGTGAGCAGTGTAGACATTTCATTGTCATACCATGAAACAACTTTANTAATGATTCCCA
TCAACAGTTTGTACTTTAGTTTGAGTAGCATCGAATAATGAACCGAAAGACATACCAACGATATCAGATGATACGATT
GGATCTTCAGTGTAACCATATGAATCATTAGCTGCAGCTTTCATTACTGAGTTAATTTCTTCTACTGAAGTTTCTTTATTA
AGAACTGCTACTAATTCTGTTACTGAACCTGTTGGAACTGGTACACGTTGTGCAGCACCGTCAAAGTTTACCATTTAAT
TCAGGGATAACAAGACCGATTGCTTTAGCAGCACCAGTTGAGTTAGGAACAATATTGTTAGCACCAGCACGGGCACG
ACGTAAGTCACCACCACGGTGAGGTCCATCAAGAAGCATTTGATCACCAGTGTAAGCGTGGATTGTAGTCATTAAACC
TTGTTTTACGCCAAAGTTATCTTGTAAAGCTTTAGCCATTGGAGCTAAACAGTTTGTAGTACATGAAGCACCTGAAATA
ACTGTTTCAGTTCCATCAAGGATATCATGGTTAGTGTTAAATACAACTGTTTTCACGTCATCTCCACCAGGAGCAGTGA
TAACAACTTTTTTAGCACCATTTTCATGTAAATGTTTTTCAGCAGCTGCTTTTTTAGCAAAGAAACCAGTTGCTTCAAGA
ACGATTTCAACACCGTCAGTTGCCCAGTCAATTTGTTCTGGATCTTTTTCAGCAGAAACTTTAATGAATTTTCCGTTAAC
GCCAAATCCACCATCTTTAACTTGTACAGTACCGTCA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Streptococcus parauberis KCTC 11537, complete genome	Streptococcus p...	1465	1465	99%	0.0	98.79%	2143887	CP002471.1
☑	Streptococcus parauberis strain SPOF3K chromosome, complete genome	Streptococcus p...	1459	1459	99%	0.0	98.67%	2128740	CP025420.1
☑	Streptococcus parauberis glyceraldehyde-3-phosphate dehydrogenase gene, complete cds	Streptococcus p...	1459	1459	99%	0.0	98.67%	1011	AF421901.1
☑	Streptococcus uberis strain SS131025-3 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete...	Streptococcus u...	1177	1177	99%	0.0	92.48%	1011	KU588171.1
☑	Streptococcus uberis strain SS131025-2 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete...	Streptococcus u...	1177	1177	99%	0.0	92.48%	1011	KU588170.1
☑	Streptococcus uberis strain SS131025-1 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, complete...	Streptococcus u...	1177	1177	99%	0.0	92.48%	1011	KU588169.1

Isolate N. 15 (10255) - *Streptococcus uberis* (Identity ID 99.67%)

GTGCAGTGTAAGACATTTCGTTGTCATACCATGAAACAACTTTAACTAATTGATTTCCATCAACAGTTTGTACTTTAGTT
TGAGTAGCATCAAACAATGAACCGTAAGCCATACCGATGATATCAGAAGATACGATTGGGTCTTCAGTGTATCCGTAT
GAATCGTTTGCAGCTGCTTTCATTGCTGCGTTGATTTCTTCAACTGAAGTTTCTTTTTCAAGAACTGCTACTAATTCAGTT
ACTGATCCAGTTGGAACAGGAACACGTTGTGCAGCACCGTCAAGTTTACCATTTAATTCTGGGATTACAAGACCGATT
GCTTTAGCAGCACCAGTTGAGTTAGGAACAATGTTGTTTGCACCAGCACGAGCACGACGAAGGTCACCACCACGGTG
TGGTCCGTCAAGGATCATTTGGTCACCAGTGTAAGCGTGGATAGTTGTCATCAAACCTTGTTTAACACCAAAGTTATCT
TGTAAAGCTTTAGCCATTGGAGCTAAACAGTTAGTAGTACATGAAGCACCTGAAATTACAGTTTCTGTACCGTCAAGA
ATGTCATGGTTTGTGTTAAATACAACAGTTTTAACATCATCTCCACCAGGAGCTGTGATAACAACTTTTTTAGCACCGTT
AGCATGTAAATGTTTTTCAGCAGCTGCTTTTTTAGCAAAGAAACCAGTTGCTTCAAGAACGATTTCTACACCGTCAGTT
GCCCAGTCAATGTTTTCTGGATCTTTTTCAGCAGAAACTTTGATGAAGTTTCCGTTAACTTCGAATCCACCATCTTTAAC
TTCAACTGTACCGTCGAAACGACCTTGAGTTGTATCATATTTCAACAAGTGTGCAAGCATATTTGGGTCAGTAAAATCG
TTAATA

	Description	Scientific Name	Max Score	Total Score	Query Cover	E value	Per. Ident	Acc. Len	Accession
☑	Streptococcus uberis strain NCTC4674 genome assembly, chromosome: 1	Streptococcus u...	1679	1679	99%	0.0	99.78%	2024265	LS483408.1
☑	Streptococcus uberis strain NZ01 chromosome, complete genome	Streptococcus u...	1679	1679	99%	0.0	99.78%	1863842	CP022435.1
☑	Streptococcus uberis 0140J complete genome	Streptococcus u...	1674	1674	99%	0.0	99.67%	1852352	AM946015.1
☑	Streptococcus uberis glyceraldehyde-3-phosphate dehydrogenase gene, complete cds	Streptococcus u...	1674	1674	99%	0.0	99.67%	1011	AF421900.1
☑	Streptococcus uberis strain NCTC3858 genome assembly, chromosome: 1	Streptococcus u...	1668	1668	99%	0.0	99.56%	1975601	LS483397.1
☑	Streptococcus uberis strain FSL Z2-047 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, partial cds	Streptococcus u...	1522	1522	90%	0.0	99.88%	827	GU392486.1
☑	Streptococcus uberis strain FSL Z2-191 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, partial cds	Streptococcus u...	1517	1517	90%	0.0	99.76%	827	GU392493.1
☑	Streptococcus uberis strain FSL Z2-262 glyceraldehyde-3-phosphate dehydrogenase (gapC) gene, partial cds	Streptococcus u...	1517	1517	90%	0.0	99.76%	827	GU392495.1

Part II: Systematic review as a tool to provide a general overview of the implementation of milk proteins as mastitis markers in dairy ruminants

On the ruminant host side, I have been working on a systematic review for understanding, using a literature search, the implementation of different host protein biomarkers for diagnosing mastitis in ruminants by using immunoassays with the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. Our goal was to report systematically and organically what has been published in the scientific literature with the integration of meta-analysis where possible.
This work was carried out with Ph.D. student Anna Giagu (AG) and Dr. Simone Dore (SD) of Istituto Zooprofilattico Sperimentale della Sardegna/Centro di Referenza Nazionale Mastopatie Ovini e Caprini (C.Re.N.M.O.C). We used "the PRISMA 2009 flow diagram" for the literature search. We examined three databases (i.e., MedLine, Scopus, and Web of Science (WoS) until January 28, 2021. For Scopus searches, we applied the default search settings (article title, abstract, and keywords), whereas in WoS we used the specific database "Web of Science Core Collection". Given the type of our systematic review, the search terms included the words: biomarker, marker, IMI, mastitis, and milk. Concerning markers, the ones most associated with the words "milk" and "mastitis" were amyloid A, haptoglobin, lactoferrin, and cathelicidin, and the two immunoassays most frequently used for measuring protein markers devoid of intrinsic enzymatic activity were ELISA and lateral flow/immunochromatography. We then combined the search terms and their related Mesh terms into 42 specific searches (Figure 16). Three researchers (AG, ST, and MP) independently screened the title, abstract, and full-text and solved disagreements by discussion and consensus. When necessary, a fourth researcher with a long publication record and expertise in the field (MFA) was consulted to reach an inclusion/exclusion decision. To synthesize the results, we applied the *"Synthesis Without Meta-analysis"* (SwiM) guidelines using tables and graphs. Based on pre-defined inclusion/exclusion criteria and selected keywords, 523 manuscripts were extracted from three databases. Of these, 33 passed the duplicate removal, title, abstract, and full-text screening for conformity to the review question and document type: 78.8% studied cows, 12.1% sheep, 9.1% goats, and 6.1% buffaloes (some included more than one dairy species). The most cited protein was M-SAA (48.5%), followed by HP (27.3%), CATH (24.2%), and LF (21.2%). The high heterogeneity among the studies in patient selection, index test, and standard reference test resulted in a data collection not amenable to meta-analysis. During the searching phase, several aspects of interest emerged, including the need to carefully define and select titles and keywords when preparing a scientific article to enable its easy retrieval from electronic databases. Also relevant is the recommendation for further review studies to carefully identify the search words that address the research question to ensure the maximum possible

coverage, as some might be unexpectedly absent or not obvious. The article has been published as Giagu A., Penati M., Traini S., Dore S., Addis M.F. Milk proteins as mastitis markers in dairy ruminants - a systematic review. Vet Res Commun. 2022 Jun;46(2):329-351. doi: 10.1007/s11259-022-09901-y. Epub 2022 Feb 23. PMID: 35195874; PMCID: PMC9165246

	Object (OR)	**Matrix**	**Diagnosis (OR)**	**Assay (OR)**
KW	*- biomarker - marker - amyloid - haptoglobin - cathelicidin - lactoferrin*	*milk*	*- "intramammary infection" - Mastitis*	*- immunoassay - ELISA - "lateral flow" - immunochromatography*
I.	Marker	milk	mastitis	immunoassay
II.	Biomarker	milk	mastitis	immunoassay
III.	Amyloid	milk	mastitis	immunoassay
IV.	Haptoglobin	milk	mastitis	immunoassay
V.	Cathelicidin	milk	mastitis	immunoassay
VI.	Lactoferrin	milk	mastitis	immunoassay
VII.	Marker	milk	mastitis	ELISA
VIII.	Biomarker	milk	mastitis	ELISA
IX.	Amyloid	milk	mastitis	ELISA
X.	Haptoglobin	milk	mastitis	ELISA
XI.	Cathelicidin	milk	mastitis	ELISA
XII.	Lactoferrin	milk	mastitis	ELISA
XIII.	Marker	milk	mastitis	"lateral flow"
XIV.	Biomarker	milk	mastitis	"lateral flow"
XV.	Amyloid	milk	mastitis	"lateral flow"
XVI.	Haptoglobin	milk	mastitis	"lateral flow"
XVII.	Cathelicidin	milk	mastitis	"lateral flow"
XVIII.	Lactoferrin	milk	mastitis	"lateral flow"
XIX.	Marker	milk	mastitis	immunochromatography
XX.	Biomarker	milk	mastitis	immunochromatography
XXI.	Amyloid	milk	mastitis	immunochromatography
XXII.	Haptoglobin	milk	mastitis	immunochromatography
XXIII.	Cathelicidin	milk	mastitis	immunochromatography
XXIV.	Lactoferrin	milk	mastitis	immunochromatography
XXV.	Marker	milk	"intramammary infection"	immunoassay
XXVI.	Biomarker	milk	"intramammary infection"	immunoassay
XXVII.	Amyloid	milk	"intramammary infection"	immunoassay
XXVIII.	Haptoglobin	milk	"intramammary infection"	immunoassay
XXIX.	Cathelicidin	milk	"intramammary infection"	immunoassay
XXX.	Lactoferrin	milk	"intramammary infection"	immunoassay
XXXI.	Marker	milk	"intramammary infection"	ELISA
XXXII.	Biomarker	milk	"intramammary infection"	ELISA
XXXIII.	Amyloid	milk	"intramammary infection"	ELISA
XXXIV.	Haptoglobin	milk	"intramammary infection"	ELISA
XXXV.	Cathelicidin	milk	"intramammary infection"	ELISA
XXXVI.	Lactoferrin	milk	"intramammary infection"	ELISA
XXXVII.	Marker	milk	"intramammary infection"	"lateral flow"
XXXVIII.	Biomarker	milk	"intramammary infection"	"lateral flow"
XXXIX.	Amyloid	milk	"intramammary infection"	"lateral flow"
XL.	Haptoglobin	milk	"intramammary infection"	"lateral flow"
XLI.	Cathelicidin	milk	"intramammary infection"	"lateral flow"
XLII.	Lactoferrin	milk	"intramammary infection"	"lateral flow"

Figure 16: Organization of the keywords by category and combinations used for the database searches (Reproduced from Giagu et al., 2022)

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Milk proteins as mastitis markers in dairy ruminants - a systematic review

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Abstract

Mastitis is one of the most impactful diseases in dairy farming, and its timely and specific detection is of great importance. The clinical evaluation of udder and mammary secretions is typically combined with the milk Somatic Cell Count (SCC) and often accompanied by its bacteriological culture to identify the causative microorganism. Several non-enzymatic milk proteins, including milk amyloid A (M-SAA), haptoglobin (HP), cathelicidin (CATH), and lactoferrin (LF), have been investigated as alternative biomarkers of mastitis for their relationship with mammary gland inflammation, and immunoassay techniques have been developed for detection with varying degrees of success. To provide a general overview of their implementation in the different dairy species, we carried out a systematic review of the scientific literature using the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analysis) guidelines. Our review question falls within the "Diagnostic test accuracy questions" and aims to answer the diagnostic question: "*Which are the diagnostic performances of mastitis protein biomarkers investigated by immunoassays in ruminant milk?"*. Based on 13 keywords combined into 42 searches, 523 manuscripts were extracted from three scientific databases. Of these, 33 passed the duplicate removal, title, abstract, and full-text screening for conformity to the review question and document type: 78.8% investigated cows, 12.1% sheep, 9.1% goats, and 6.1% buffaloes (some included more than one dairy species). The most frequently mentioned protein was M-SAA (48.5%), followed by HP (27.3%), CATH (24.2%), and LF (21.2%). The large amount of heterogeneity among studies in terms of animal selection criteria (45.5%), index

test (87.9%), and standard reference test (27.3%) resulted in a collection of data not amenable to meta-analysis, a common finding illustrating how important it is for case definitions and other criteria to be standardized between studies. Results are presented according to the SWiM (Synthesis Without Meta-analysis) guidelines. We summarized the main findings reported in the 33 selected articles for the different markers and report their results in comparative tables, including sample selection criteria, marker values, and diagnostic performances, where available. Finally, we report the study limitations and bias assessment findings.

Keywords: immunoassay, intramammary infection, amyloid A, haptoglobin, cathelicidin

Introduction

As a critical factor affecting milk yield and quality, mastitis represents the most relevant health problem in dairy ruminants worldwide (Ruegg 2017). According to the National Mastitis Council (NMC), mastitis is defined as *"an inflammation of one or more quarters/halves of the mammary gland, almost always caused by an infecting microorganism"* (Lopez-Benavides et al. 2012). Clinical mastitis can be diagnosed by examination of the udder and of the milk for visible abnormalities, identifying subclinical mastitis is more challenging (Menzies and Ramanoon 2001; Oliver et al. 2004). In animals with subclinical mastitis, the diagnosis is performed mainly on the milk through indirect methods such as the Somatic Cell Count (SCC) (Bergonier et al. 2003; Persson and Olofsson 2011) or its field version, the California Mastitis Test (CMT) (Kelly et al. 2018). Being typically caused by an intramammary infection (IMI) (Ezzat Alnakip et al. 2014), the disease is also investigated through direct methods such as the bacteriological culture (BC) (Contreras et al. 2007) or molecular assays (i.e., PCR) (Chakraborty et al. 2019). The indirect screening approaches rely mainly on the principle that the udder microenvironment changes during the inflammatory process, with an increase in the concentration of immune cells and immune mediators (Hughes and Watson 2018). Polymorphonuclear neutrophils (PMNs) are the prevalent immune cells in the acute phase of mastitis; SCC and CMT perform well as diagnostic tools because of their indirect relationship to the presence of PMNs (Leitner et al., 2000; Sordillo and Streicher 2002). These tests may lack specificity (Rossi et al. 2018), especially in small ruminants (Souza et al. 2012). BC lacks sensitivity (Chakraborty et al. 2019), and is not particularly useful as a mastitis screening tool given its time, labor, and cost requirements. Clinical examination, SCC, CMT, and BC, should be combined to increase diagnostic performance (Lam et al. 2009; Chakraborty et al. 2019); a universally accepted specific diagnostic algorithm or protocol is not yet available. During mammary gland inflammation, numerous antibacterial and immune defense proteins, including Acute Phase Proteins (APPs),

lactoferrin (LF), cathelicidins (CATH), cytokines, chemokines, and growth factors, are released in the milk and can potentially serve as "mastitis markers" (Smolenski et al. 2011; Thomas et al. 2015). Their implementation as alternative/integrative diagnostic tools has been the subject of several studies (Viguier et al. 2009). Many focused on discovering new biomarkers for implementing diagnostic tools with improved sensitivity and specificity compared to the currently available assays. The measurement methods for inflammation-related proteins devoid of intrinsic enzymatic activity are typically immunoassays employing highly specific antibodies (Viguier et al. 2009). Adding to the possibility of increased diagnostic performances, the integration of traditional diagnostic approaches with immunoassays measuring mastitis marker proteins might bring additional benefits, including the ability to work efficiently on frozen samples, high analytical throughput, relatively low analytical costs, the minimal requirements for dedicated personnel training, and specialized or expensive instrumentations (Addis et al. 2016a). A group of widely investigated potential biomarkers are Acute Phase Proteins (APPs), commonly employed as clinical biomarkers of inflammation in serum but also found in milk. In particular, the milk isoforms of serum amyloid A (M-SAA) and haptoglobin (HP) (Hussein et al. 2018; Chakraborty et al. 2019; Iliev and Georgieva 2019;) are among those most used. Other proteins indicated as suitable mastitis markers are lactoferrin (LF) (Shimazaki and Kawai 2017) and cathelicidins (CATH) (Smolenski et al. 2011). Biomarker discovery and implementation are constantly evolving, and comparative data on their diagnostic performances are lacking. It is difficult to establish their relative advantages in the different dairy ruminant species compared to the current diagnostic approaches. To provide an organic overview of the topic, to understand if the data currently available in the literature are amenable to meta-analysis, and to attempt a comparative assessment of the respective diagnostic performances, we carried out a literature survey using the systematic review approach based on the PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. In veterinary medicine, the methodology for systematic reviews has been defined by Sargeant and O'Connor (2020), who identified specific steps to follow. Our review question falls within the fourth type, "Diagnostic test accuracy questions", aimed at summarizing diagnostic test accuracy. Specifically, this systematic review examines the scientific literature to answer the diagnostic question: "Which are the diagnostic performances of mastitis protein biomarkers investigated by immunoassays in ruminant milk?".

Methods

Information sources and search strategy

We carried out this systematic review according to the guidelines of the PRISMA statement (Moher et al. 2009). We searched three databases (i.e., MedLine, Scopus, and Web of Science) through January 28, 2021. For Scopus searches, we applied the default search settings (Article title, abstract, and keywords), in Web of Science, we used the specific database "Web of Science Core Collection". Our review question falls within the fourth type, *"Diagnostic test accuracy questions"*, aimed at summarizing diagnostic test accuracy and at answering the diagnostic question: "*Which are the diagnostic performances of mastitis protein biomarkers investigated by immunoassays in ruminant milk?"* as suggested by Sargeant and O'Connor (2020) for systematic reviews in veterinary medicine. The search terms included the words "biomarker", "marker," "intramammary infection", "mastitis", and "milk". These search terms were enriched with the most common markers and detection assays to improve the retrieval of relevant scientific articles. Concerning markers, an initial literature survey indicated that the ones most associated with the words "milk" and "mastitis" were M-SAA, HP, LF, and CATH. The two immunoassays most frequently used for measuring protein markers devoid of intrinsic enzymatic activity were ELISA and lateral flow/ immunochromatography. Once defined, we combined the search terms and their related Mesh terms into 42 specific searches, as follows: ("biomarker" OR "marker" OR "amyloid" OR "haptoglobin" OR "cathelicidin" OR "lactoferrin") AND ("intramammary infection" OR "mastitis") AND ("milk") AND ("immunoassay" OR "ELISA" OR "lateral flow" OR "immunochromatography") (Supplementary Table I).

Study selection, data extraction, and synthesis method

Three researchers (AG, ST, and MP) independently screened the title, abstract, and full-text to assess the article's compliance with the review question and resolved disagreements by discussion and consensus. When necessary, a fourth researcher with expertise in the field (MFA) was consulted to reach an exclusion decision. Adding to the articles not relating to the review question, we excluded those written in languages different from English and belonging to the categories review, case report, report, book chapter, editorial, abstract, and letter. From each eligible document, the following data were extracted: species, first author, year, country, study design, biomarker, technique, sample type and size, SCC, pathogens, unit of measurement, results, sensitivity, specificity, and cut-off. To synthesize the results, we applied the *"Synthesis Without Meta-analysis"* (SWiM) guidelines (Campbell et al. 2020) by using tables and graphs.

Quality assessment

The tool consists of 14 questions and two main sections, bias assessment and applicability, including four and three key domains. In bias assessment, for every study, were assessed the “animal selection” strategy, the “index test”, the “reference standard”, and “flow and timing”. The term "index test" is referred to the test object of study, while "reference standard" refers to the standard test considered the best available test to diagnose the disease of interest (i.e. a single test, follow-up, or combination of tests). We collected and rated how much the studies matched the review question in the applicability assessment. For both sections, the risk was expressed as “high”, “low”, and “unclear” risk when data were insufficient. The 33 screened records showed high heterogeneity in study design, animal selection, and standard reference tests.

Results and discussion

Results of the PRISMA procedure

The steps of the literature search are summarized in the PRISMA 2009 flow diagram (Figure 1). The search led to the identification of 507 scientific papers (220 MedLine + 131 Scopus + 156 Web of Science); 16 further records were then added to the original search through an expert revision of the literature, resulting in 523 manuscripts (Supplementary Tables II, III, IV, and V). After removing duplicates, 133 records entered three main screening steps. Records were screened on the title, secondly on the abstract (n = 72, intermediate step not included in Fig.1), and finally on the full-text for evaluating the eligibility to qualitative and quantitative analysis (Supplementary Tables VI and VII). As a result of this procedure, 33 scientific articles were considered eligible (Supplementary Table VIII).

Species overview

By sorting the number of papers based on the dairy species, out of 33 manuscripts, 26 (78.8%) investigated cows, 4 (12.1%) sheep, 3 (9.1%) goats, and 2 (6.1%) buffaloes (Table 1). The number of records does not match because two papers addressed more than one species.

Cow. Out of 26 papers on cow milk, 15 (57.7%) investigated M-SAA, 9 (34.6%) HP, 5 (19.2%) LF, 2 CATH (7.7%), interleukin 1β (IL1β) and interleukin 6 (IL-6). Other biomarkers were Alpha-1-Acid Glycoprotein (AGP), bovine serum albumin (BSA), C-reactive protein (CRP), immunoglobulin G (IG), interleukin 8 (IL8), interleukin 10 (IL10), interleukin 12 (IL12) lipopolysaccharide-binding protein (LBP), Transforming Growth Factor α (TGFα), Transforming Growth Factor β (TGFβ), and Tumor Necrosis Factor α (TNFα). They were addressed in 1 paper each (3.8%) (Table 1). The samples

were represented by quarter milk in 18/26 (69.2%) and by composite milk in 8/26 (30.8%). In one record (Sobczuk-Szul et al. 2014), the milk sample type was not specified, in another study (Thomas et al. 2015), both quarter and composite samples were used. Concerning the diagnostic methods, ELISA was used in 25 (96.2%) records, in 1 paper (3.8%), the biomarker was investigated by SPARCL. We observed 25 (96.2%) observational studies related to natural inflammation/infection and only one experimental infection study. Tables 2, 3, 4, and 5 summarize the main findings of the 26 papers evaluating cows.

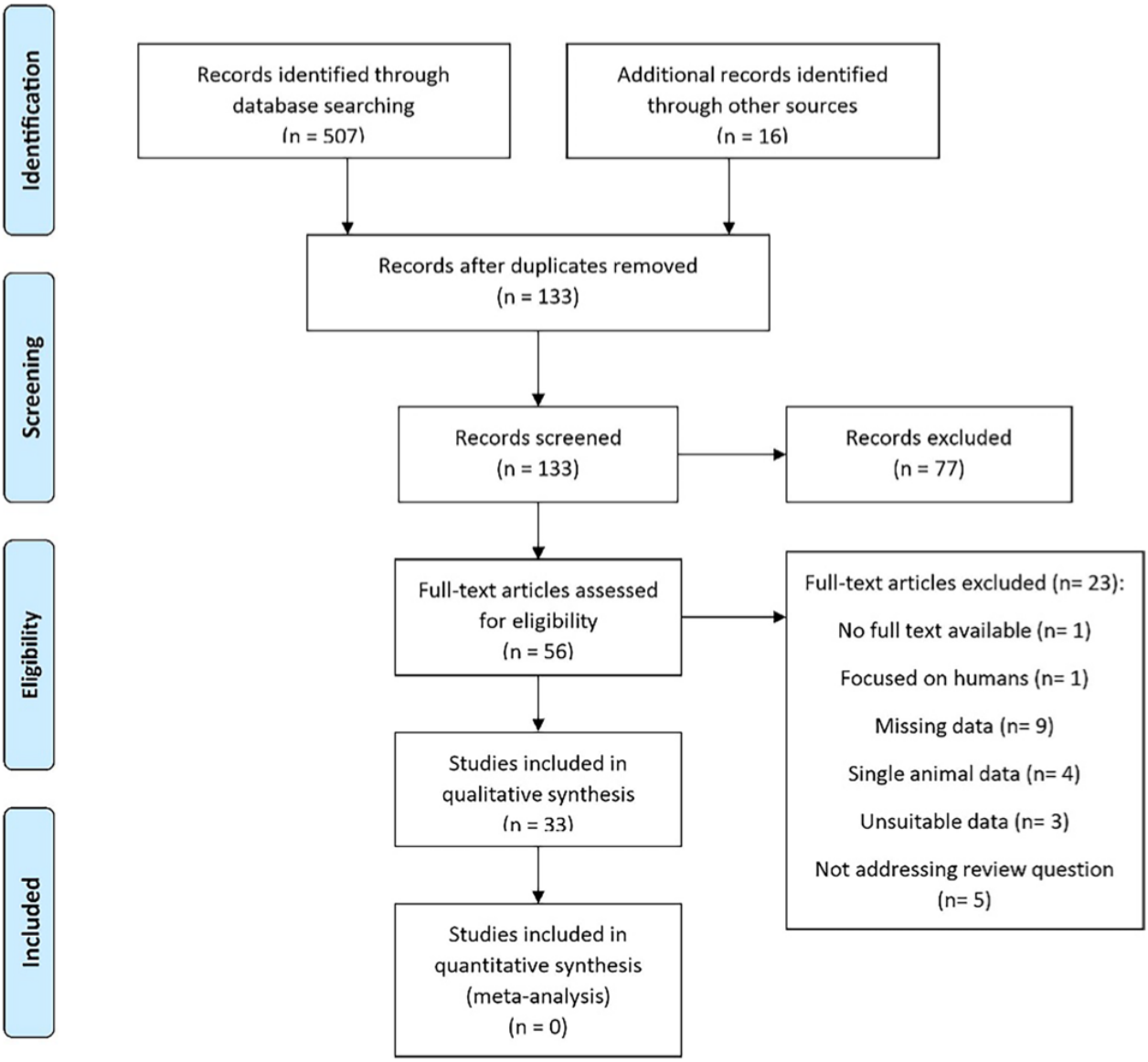

Figure 1: PRISMA 2009 flow diagram

Sheep. Out of 4 papers on sheep milk, 2 (40.0%) assessed CATH, while one each (20.0%) was on interleukins and M-SAA, respectively. ELISA was used in all studies, three of which were observational (60.0%) and 2 (40.0%) experimental. All studies were carried out on half-udder milk samples. Table 6 summarizes the main findings of the 4 papers.

Goat. Two (66.7%) out of 3 studies assessed CATH, while 1 (33.3%) assessed LF. ELISA was used in all studies, which were all observational. All papers investigated biomarkers from half-udder, but one (Chen et al. 2004) also used bulk milk samples. Table 7 summarizes the main findings of the three reports.

Water buffalo. Only two observational studies were performed on buffalo. The biomarkers investigated were LF and CATH from quarter milk by ELISA. Table 8 summarizes the main findings of the two papers.

Biomarker overview

Table 1 summarizes our results presented in descending order of records addressing biomarkers and dairy species. Among all markers, M-SAA was the most frequently mentioned (n. 16; 48.5%), followed by HP (n. 9; 27.3%;), CATH (n. 8; 24.2%), and LF (n. 7; 21.2%;). Other markers investigated were IL1β and IL6, addressed in 3 papers each (9.1%), followed by IgG (n. 2; 6.1%), and finally AGP, BSA, CRP, IL8, IL10, IL12, LBP, TGFα, TGFβ, TNFα (n. 1; 3.0%).

Table 1: Species and biomarker overview

Biomarker	%	N°	Cow	Sheep	Buffalo	Goat
M-SAA	48.5	16/33	15	1	-	-
HP	27.3	9/33	9	-	-	-
CATH	24.2	8/33	2	2	1	3
LF	21.2	7/33	5	-	1	1
IL1β	9.1	3/33	2	1	-	-
IL6	9.1	3/33	2	1	-	-
AGP	3	1/33	1	-	-	-
BSA	3	1/33	1	-	-	-
CRP	3	1/33	1	-	-	-
IgG	3	1/33	1	-	-	-
IL8	3	1/33	-	1	-	-
IL10	3	1/33	1	-	-	-
IL12	3	1/33	1	-	-	-
LBP	3	1/33	1	-	-	-
TGFα	3	1/33	1	-	-	-
TGFβ	3	1/33	1	-	-	-
TNFα	3	1/33	1	-	-	-

Acronyms: M-SAA, milk amyloid A. HP, haptoglobin. CATH, cathelicidins. LF, lactoferrin. IL1β, interleukin 1β. IL6, interleukin 6. AGP, Alpha-1-Acid Glycoprotein. BSA, bovine serum albumin. CRP, C-reactive protein. IgGs, immunoglobulin G. IL8, interleukin 8. IL10, interleukin 10. IL12, interleukin 12. LBP, lipopolysaccharide-binding protein. TGFα, Transforming Growth Factor α. TGFβ, Transforming Growth Factor β. TNFα, Tumor Necrosis Factor α. %, percent of articles relating to the numbers of papers addressing the biomarker out of the total. N°, number of articles relating to specific biomarker out of the 33 eligible records

Milk serum amyloid (M-SAA) M-SAA is produced extrahepatically by healthy mammary epithelial cells (McDonald et al. 2001; Larson et al. 2005) and during inflammation (Grönlund et al. 2003; Larson et al. 2005; Brenaut et al. 2014). M-SAA was the protein most investigated as a subclinical mastitis marker in ruminant milk, particularly in dairy cows (Table 2).We observed that in 17 papers, M-SAA was investigated predominantly by ELISA with the commercial kit Tridelta solid sandwich ELISA in two variants (Tridelta Mast ID range MAA assay, Tridelta Development Ltd., Kildare, Ireland, Cat. No.: TP-802 for serum and TP-807 for milk). To diagnose mastitis, the authors did not discriminate between serum or milk amyloid isoforms but for the different matrices, defining the protein as SAA when analyzing serum and M-SAA when analyzing milk. In 5 studies, M-SAA was investigated only by TP-802 (Grönlund et al. 2005; Eckersall et al. 2006b; Kováč et al. 2007; Åkerstedt et al. 2007, 2009), in 5 only by TP-807 (Åkerstedt et al. 2011; Shirazi-Beheshtiha et al. 2011; Jaeger et al. 2017; Hussein et al. 2018; Bochniarz et al. 2020; Wollowski et al. 2021), in 2 by both TP-802 and TP-807 (Gerardi et al. 2009; Safi et al. 2009) and in 5 a Tridelta kit was used but the test category was unspecified (Suojala et al. 2008; Pyörälä et al. 2011; Kovačević-Filipović et al. 2012; Szczubiał et al. 2012; Thomas et al. 2015). Gerardi et al. (2009) investigated M-SAA in milk with both TP-807 and TP-802 assays to compare their diagnostic performances. The sensitivity of the TP-807 test is 0.10 μg/ml, but a cut-off able to discriminate healthy from mastitic milk has not yet been defined Miglio et al. (2013) reported a M-SAA peak almost ten times higher in sheep milk than cow milk. Although no official reference range is fixed for M-SAA in milk, healthy sheep milk concentration ranges from 23.75 to 35.61 μg/ml (Miglio et al. 2013), higher than that observed in cow milk (range: 0.0 - 7.5 μg/ml) (Gerardi et al. 2009). In goats, the MAA as mastitis marker was not suitable. In this species, M-SAA levels increase physiologically as lactation progresses, as does SCC, even in the absence of infection (Pisanu et al. 2020).

Haptoglobin (HP) HP was the second most represented marker in our literature search. Its performance for mastitis detection was analyzed in 9 records, only for cows and by ELISA (Table 3). HP found in milk has an undefined origin like M-SAA, extrahepatic production may also occur in the mammary tissue. It has been demonstrated that HP concentration increases in milk upon endo- toxin challenge, experimental, and natural intramammary infection (IMI) (Grönlund et al. 2003; Eckersall et al. 2006; Gerardi et al. 2009). HP appears in milk and rises in level at 3 hours and in blood 9 hours after inflammation (Hiss et al. 2004), indicating that the production of this biomarker by the mammary gland is rapid and specific. Various authors' diagnostic performance reported in cows is promising (Table 3) and encourages its evaluation in other dairy species. For its characteristics, this biomarker might also be promising for diagnosing caprine mastitis, particularly in late lactation, when the SCC is high, and other markers fail to provide satisfactory performances (Pisanu et al. 2020).

Cathelicidin (CATH) CATH was measured mainly by ELISA in goats (n. 3), cows (n. 2), sheep (n. 2), and water buffalo (n. 1). CATH are host defense proteins with antimicrobial and immunomodulatory functions (Van Harten et al. 2018) produced by milk PMNs (Kościuczuk et al. 2012) and mammary epithelial cells (Zanetti 2004, 2005; Addis et al. 2013; Cubeddu et al. 2017). The ruminant genome contains numerous CATH proteoform genes, but their differential abundance in mastitic milk is poorly known (Zanetti 2005). CATH showed a high diagnostic performance, especially in cows and sheep, also in late lactation. Using a threshold set with negative healthy controls, a good sensitivity of the dedicated ELISA is reached not only for cow and sheep milk (Addis et al. 2016a, 2016b) but also for water buffalo milk (Puggioni et al. 2020a). Applying CATH-ELISA in goats remains unsatisfactory in late lactation, especially in pluriparous goats. The related physiological increase in PMN compromises its reliability, for M-SAA (Pisanu et al. 2020).

Lactoferrin (LF) LF was primarily detected by ELISA in studies involving cows (n. 5), goats (n. 1,) and water buffalo (n. 1). LF is a glycoprotein of the immune defense secreted by mammary epithelial cells during the late stage of milking and mammary involution (Welty et al. 1976; Galfi et al. 2016a). The presence of LF in milk is due to secretion by epithelial cells and degranulation of PMNs during inflammation (Lash et al. 1983). Even though LF is not an APP, it increases remarkably during the inflammatory response due to its production by mammary epithelial cells (Galfi et al. 2016a). Concerning test characteristics for goats and cows, two studies carried out a competitive ELISA by using a lactoferrin antiserum from rabbits, and goat lactoferrin was isolated and purified (Chen and Mao 2004; Chen et al. 2004). In other studies, cow LF was quantified by a commercial sandwich LF ELISA kit (Bethyl Laboratories, Montgomery, TX) (Cheng et al. 2008; Sobczuk-Szul et al. 2014; Galfi et al. 2016a, 2016b). For water buffalo, a specific ELISA kit was produced for the study (Özenç et al. 2019). None of the studies reported test characteristics for LF, and no information on sensitivity or specificity is available for this marker. Other markers, IL1β and IL6, were studied in both cows and sheep (Tab.2), IL-8 only in sheep, and the other proteins (AGP, BSA, CRP, IG, IL10, IL12, LBP, TGFα, TGFβ, TNFα) only in cows. In humans, immune cytokines such as TNFα, INFγ, and ILs are investigated as inflammatory markers to detect subclinical mastitis and identify Th1/Th2 ratio in the inflammatory process (Tuaillon et al. 2017). CRP was studied as a predictor of the severity of symptomatology in women's breast inflammation (Fetherston et al. 2006). In cows, immune cells and their related cytokines have been the subject of studies (Gulbe et al. 2020; Shaheen et al. 2020), especially pro-inflammatory immune mediators. In other dairy ruminants, these proteins and their roles in mastitis need to be studied.

Table 2: Cow results obtained for M-SAA by applying ELISA and SPARCL (Dalanezi et al. 2020). The unit of measurement is µg/mL*

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cut-off
Kováč et al. 2007			*Composite*			*Median / Range*			
	<100			SCC group 1	5	0.67 / 0-1.79			
	100-400			SCC group 2	5	1.52 / 0-7.15			
	>400			SCC group 3	8	26.54 / 1.81-54.28			
Suojala et al. 2008			*Quarter*		*7*	*Mean (SD)*			
		E. coli		Clinical		1.32 (0.95)			
Åkerstedt et al. 2009	*Mean (SD)*		*Composite*		*68*	*Mean (SD)*			
	218 (179)					1.12 (1.16)			
Gerardi et al. 2009	*Mean / Median*		*Quarter*			*Mean / Median*			
	618 / 163			Subclinical (TP-802)	40	9.8 / 2.3			
				Subclinical (TP-807)		5.5 / 0.5			
	2,704 / 1,120			Clinical (TP-802)	24	16.1 / 8.0			
				Clinical (TP-807)		6.9 / 3.8			
	58 / 28			Healthy (TP-802)	4	0.5 / 0.4			
				Healthy (TP-807)		0.1 / 0.1			
Safi et al. 2009	*Mean (SD) / Median / Range*		*Quarter*			*Mean (SD) / Median / Range*			
	5,000 (9,500) /1,250 / 3-51,840	*S. aureus*			106	67 (120) / 28 / 14-843			
	93 (68)/75 / 3-266	Negative		Healthy	134	9 (5) / 8 / 1-29			
							90.6	98.3	16.4
Pyörälä et al. 2011			*Quarter*			*Median (IQR)*			
				Subclinical	136	13.4 (3-83.5)			
				Clinical	98	22.7 (4.4-102.5)			
		S. aureus			44	16.4 (3.5-80.2)			
		NAS			45	4.4 (1.6-70.5)			
		S. uberis			43	21.2 (5.2-99.5)			
		S. dysgalactiae			48	23.9 (7.7-85.3)			
		E. coli			23	279.5 (19.7-675.0)			
		A. pyogenes			24	3.0 (<0.3-41.8)			
		Other			7	41.3 (3.9-720.0)			
Kovačević-Filipović et al. 2012	*Mean (SD) /Range*		*Quarter*			*Mean (SD) /Range*			
	20 (2) / 7-31	Negative		Control group	10	1.4 (1.1) / 0-4.15			
	2,066 (28) / 556-3,617	*S. aureus*		Subclinical	10	102.6 (88.8) / 27.9-324.5			
Shirazi-Beheshtiha et al. 2011			*Quarter*			*Mean (SD) / Median / Range*			
	<130	Negative		Healthy	38	0.61 (0.5) / 0.44 / 0.1-1.9			
	>130	Positive		Subclinical	52	12.83 (12.8) / 7.88 / 0.84-50.5			
							92.3	92.1	1.6
Szczubiał et al. 2012			*Quarter*			*Mean (SD) /Range*			

Table 2: (Continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cut-off
		S. aureus		Subclinical	12	41.75 (26.6) / 8.76-74.75			
		S. agalactiae			9	60.11 (17.53) / 9.78-111.26			
		S. dysgalactiae			18	72.43 (43.22) / 19.90-121.20			
		S. uberis			18	92.23 (28.64) / 8.58-221.64			
		NAS			19	12.47 (6.95) / 6.53-23.33			
		Candida spp.			8	101.00 (55.56) / 9.61-199.36			
		Negative		Healthy	14	11.67 (7.40) / 5.24-19.04			
Wollowski et al. 2021			*Quarter*			*Mean*			
				Subclinical	107	2.62			
				Clinical (all)	201	6.67			
				Clinical (mild)	45	6.14			
				Clinical (moderate)	95	5.69			
				Clinical (severe)	61	8.63			
				Healthy	67	1.06			
		S. aureus		Subclinical	12	4.38			
		NAS		Subclinical	14	1.57			
							65	76	1.28
							77	83	1.81
Thomas et al. 2015	*Range*		*Composite*			*Median / Range*			
	9 - 6,154			All	54	1.17 / <0.6-50.13			
	<100			Healthy	29	0.6 / <0.6-50.13			
	101 - 200			Subclinical	8	0.6 / <0.6			
	>200			Clinical	17	0.6 / <0.6-24.81			
Hussein et al. 2018			*Quarter*			*Mean*			
	≤500			SCC group 1	148	3.58			
	≥500			SCC group 2	72	35.2			
Bochniarz et al. 2020			*Quarter*			*Median / Range*			
		S. uberis		Clinical	14	2.59 / 0.47-5.84			
		S. agalactiae			7	3.88 / 0.88-7.60			
		Strep. spp.			30	1.13 / 0.42-7.60			
	<100	Negative		Healthy	10	0.32 / 0.15-0.51			
Jaeger et al. 2017	*Median (IQR)*		*Composite*			*Median (IQR)*			

Table 2: (Continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cut-off
	813 (158-2,512)	*S. aureus*			26	8.19 (1.52-49.85)			
	309 (68-1,288)	*S. uberis*			14	6.27 (0.28-30.01)			
	295 (16-3,090)	Other major			16	5.19 (0.19-14.78)			
	457 (25-2,818)	Major			56	6.68 (0.16-41.87)			
	178 (19-1,023)	NAS			76	3.24 (0.00-13.71)			
	240 (30-946)	*C. bovis*			109	3.63 (0.00-14.75)			
	257 (21-933)	NAS + *C. bovis*			28	3.60 (0.00-16.19)			
	62 (11-331)	Other minor			6	1.40 (0.42-5.16)			
	234 (18-1,047)	Minor			219	3.44 (0.00-14.68)			
	109 (10-813)	Negative			158	1.28 (0.00-14.75)			
Dalanezi et al. 2020*			*Individual*			*Median (IQR) / Range*			
		K. pneumoniae		Clinical	18	52.4 (31.9-97.1) / 2.0-178.8			
		E. coli			24	20.5 (8.6-47.2) / 0.0-264.0			
		S. aureus			15	38.2 (21.8-94.9) / 13.3-129.5			
		Strep. spp.			16	63.8 (48.4-70.6) / 21.0-151.4			
		Mycoplasma spp.			18	35.2 (17.4-51.5) / 0.0-102.0			
		Enterococcus spp.			18	55.4 (26.8-86.1) / 0.0-250.0			
		NAS			24	14.9 (8.7-37.7) / 0.0-141.7			

Table 3: Cow results obtained for haptoglobin by ELISA. The unit of measurement is µg/mL

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cutoff
Hiss et al. 2007	*Median / Range*		*Quarter*			*Median / Range*			
	40 / 4-1,512	Negative		Group 1	79	0.70 / 0.35-16.0			
	70,500 / 6-2,249	*C. bovis*		Group 2	70	1.85 / 0.35-85.0			
	136 / 12-10,000	Mixed inf.		Group 3	45	2.4 / 0.5-150.2			
	167 / 9-4,171	NAS		Group 4	60	3.1 / 0.35-576.0			
	405 / 32-10,000	*Strep.* spp.		Group 5	29	4.4 / 0.50-974.0			
	335 / 8-8,804	Mixed + *S. aureus*		Group 6	35	4.8 / 0.35-232.4			
	1,741 / 16-10,000	*S. aureus*		Group 7	49	39.6 / 0.35-304.8			
	8,861 / 1,658-10,000	*E. coli*		Group 8	3	81.0 / 59.0-184.0			
							85	92	2.2 (SCC 100)
							89	92	2.7 (SCC 200)
Kováč et al. 2007			*Composite*			*Median / Range*			
	<100			Group 1	7	0 / 0-0			
	101-400			Group 2	7	0 / 0-0.68			
	>400			Group 3	8	6.76 / 0-20.0			
Safi et al. 2009	*Median / Range*		*Quarter*			*Median / Range*			
	1,250 / 3-51,840	*S. aureus, Strep. ag.*		Subclinical	39	10 / 0-1,382			
	75 / 3-266	Negative		Healthy	134	0 / 0-500			
							90.6	68.6	3.9
Zeng et al. 2009			*Quarter*			*Mean (SD)*			
	< 250			SCC group 1	347	0.50 (0.15)			
	> 250			SCC group 2	46	7.18 (2.10)			
Wenz et al. 2010			*Quarter*			*Mean (95% CI)*			
				Clinical (mild)	87	503 (344-735)			
				Clinical (moderate-severe)	60	1,013 (644-1594)			
		Gram-negative			83	1,126 (759-1,670)			
		Gram-positive			64	575 (375-881)			
		NAS			19	403 (196-828)			
		Strep. spp.			45	686 (418-1,127)			
		E. coli			57	1,052 (675-1,639)			
		Coliforms			26	1,370 (704-2,666)			
Pyörälä et al. 2011			*Quarter*			*Median (IQR)*			

Table 3 (continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cutoff
		S. aureus			44	33.0 (<7.8-95.3)			
		NAS			45	7.8 (<7.8-73)			
		Strep. uberis			43	36.7 (<7.8-249.5)			
		E. coli			23	243.5 (32.1-625)			
		A. pyogenes			24	440.3 (164.5-961.5)			
		Strep. dysgalactiae			48	34.5 (<7.8-125.5)			
		Other bacteria			7	<7.8 (<7.8-159.5)			
				Subclinical	136	33.8 (<7.8-135.5)			
				Clinical	98	80 (10.2-332.0)			
	>200 >100						52.974.0	94.664.4	
Thomas et al. 2015	*Median / Range*					*Median / Range*			
			Quarter	All	149	3.60 / <0.4-420			
	101-200		Composite	Subclinical	8	4.02 / <0.4-5.28			
	>200			Clinical	17	6.40 / <0.4-55.46			
	96 / 9-6,154			All	54	3.46 / <0.4-55.46			
	<100			Healthy	29	2.96 / <0.4-13.74			
Dalanezi et al. 2020			*Individual*			*Median (IQR) / Range*			
		K. pneumoniae		Clinical	18	206.1 (126.3-468.1) / 0.0-1,113.3			
		E. coli			24	164.1 (71.7-305.1) / 0.0-2,009.4			
		S. aureus			15	158.7 (0.0-300.0) / 0.0-596.1			
		Strep. spp.			16	179.0 (130.2-363.7) / 0.0-812.2			
		Mycoplasma spp.			18	102.0 (0.0-332.8) / 0.0-582.9			
		Enterococcus spp.			18	43.0 (0.0-127.3) / 0.0-213.0			
		NAS			24	0.0 (0.0-66.3) / 0.0-319.1			
Wollowski et al. 2021			*Quarter*			*Mean*			

Table 3: (Continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Sample	Sample group	N	Value	%Se	%Sp	cutoff
				Subclinical	107	10.15			
				Clinical (all)	115	13.73			
				Healthy	67	0.98			
		Strep. uberis		Subclinical	22	11.1			
		S. aureus		Subclinical	12	11.86			
		NAS		Subclinical	14	8.52			
							92	94	3.65
							96	99	5.40

Method overview

Clinical signs, SCC or CMT, and bacteriological culture results were the reference standard methods used to define the presence of mastitis or IMI in dairy ruminants, in association or alone (Chakraborty et al., 2019). Among the analytical techniques applied to evaluating protein biomarkers, ELISA was used in 31 of 33 (93.9%) selected records, and SPARCL (Spatial Proximity Analyte) and RID (radial immunodiffusion) were each applied in 1 paper.

Limitations of the systematic review

Issues in research methodology Our research encountered several critical issues in applying the PRISMA standard methodology, especially concerning the search strategy. While selecting the best-performing keywords for our review, we assessed several combinations for finding those enabling the collection of the most comprehensive but selective set of publications possible. During the process, we had some unexpected findings; for instance, the keyword “ruminant” produced a less sensitive search, leading to the decision to remove it. This indicates that the word “ruminant” is uncommonly used in title, abstract, or keywords, probably because the authors prefer to report only the name of the dairy species. Misleading titles and abstracts led to identifying papers that did not address the research question, which had to be excluded (as detailed in Methods).

We compensated for the possible loss of records consequent to improper index terms with an additional critical revision of the literature performed on PubMed. The references of each retrieved article were screened as a further compensative measure. There is always a risk of exclusion for those articles that do not contain at least one of the selected search terms in the title, abstract, or keywords. The authors must take particular care when drafting these parts to maximize article retrieval.

Bias assessment and applicability of studies Defining quality assessment of primary studies is an essential step in systematic reviews. The risk of bias and applicability must be evaluated and scored in all studies, especially those focused on diagnostic accuracy. We applied QUADAS, a quality assessment tool, to all the selected studies. Concerning the risk of bias (Supplementary Table IX), on animal selection (domain 1), 18/33 (54.5%) studies had a low risk of bias, 15/33 (45.5%) high, and 0/33 (0.0%) unclear risk. Regarding the index test (domain 2), one study out of 33 (3.0%) had a low risk of bias, 29/33 (87.9%) had a high risk, and 3/33 (9.1%) had an unclear risk. For the reference standard (domain 3), we observed a low risk of bias in 22/33 studies (66.7%), high risk in 9/33 (27.3%), and unclear risk in 2/33 (6.1%). Finally, flow and timing (domain 4) showed a low risk of bias in 21/33 records (63.6%), high risk in 11/33 (33.3%), and unclear risk in 1/33 (3.0%). Many studies showed low concerns about applicability, especially regarding domain 3 (Supplementary Table X). In detail, in domain 1, low risk was reported in 29/33 (87.9%), high in 4/33 (12.1%), and unclear in 0/53 (0.0%). In domain 2, records had low risk in 25/33 (75.8%), high in 5/33 (15.1%), and unclear 3/33 (9.1%), whereas in domain three, we observed low risk in 31/33 (93.9%) papers, high in 2/33 (6.1%) and unclear in 0/33 (0.0%).

Table 4 Cow, results obtained for other non-cytokine markers.

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Assay	Unit	Sample	Sample group	N	value	%Se	%Sp	Cutoff
Dalanezi et al. 2020			*AGP*	*SPARCL*	*µg/ml*	*Individual*	*Clinical*		*Median (IQR) / Range*			
		K. pneumoniae						18	34.9 (22.4-145.3) /14.4-305.0			
		E. coli						24	47.3 (24.8-155.7) / 6.8-892.9			
		S. aureus						15	51.3 (17.3-207.4) / 11.9-289.9			
		Streptococcus spp.						16	29.7 (19.0-49.3) / 11.3-100.7			
		Mycoplasma spp.						18	24.7 (15.1-79.2) / 10.6-427.5			
		Enterococcus spp.						18	17.9 (15.8-22.2) / 10.1-297.5			
		NAS						24	9.7 (7.7-9.7) / 5.9-42.6			
Wenz et al. 2010			*BSA*	*ELISA*	*mg/mL*	*Quarter*			*Mean*			
							Clinical (mild)	87	4.34			
							Clinical (moderate-severe)	60	5.37			
		Gram-negative						83	6.08			
		Gram-positive						64	3.85			
		NAS						19	3.22			
		Streptococcus spp.						45	4.09			
		E. coli						57	6.39			
		Coliforms						26	5.38			
Addis et al. 2017	*Median (IQR)*		*CATH*	*ELISA*	*AOD450*	*Quarter*			*Median (IQR):*			
	7.5 (3-21)						Healthy	100	0.089 (0.084-0.094)			
	5,588 (2,540-7,814)						Clinical	435	11.850 (3.090-27.120)			
	6,543 (4,316-8,409)	Mixed infection						25	8.970 (3.023-30.100)			
	6,362 (4,586-7,992)	*S. agalactiae*						59	27.480 (12.290-30.100)			
	5,818 (3,748-8,665)	Other bacteria						36	6.420 (2.454-20.827)			
	5,513 (3,108-7,368)	*S. aureus*						14	16.165 (7.071-22.302)			
	5,522 (2,762-7,521)	Gram-negative						102	11.640 (4.805-22.302)			
	5,405 (2,461-7,394)	*Strep.* spp.						99	13.020 (3.727-30.000)			
	5,049 (1,106-7,992)	Negative						59	10.620 (2.803-18.920)			
	3,037 (1,078-6,146)	NAS						41	3.120 (0.866-9.055)			
										99.08	100	0.115
Wollowski et al. 2021			*CATH*	*ELISA*	*NOD450*	*Quarter*			*Mean*			

Table 4 (continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Assay	Unit	Sample	Sample group	N	value	%Se	%Sp	Cutoff
							Subclinical	107	0.951			
							Clinical (all)	115	2.420			
							Healthy	67	0.001			
		Strep. uberis					Subclinical	22	1.238			
		S. aureus					Subclinical	12	1.309			
		NAS					Subclinical	14	0.326			
										83	97	0.00
										98	99	0.053
Dalanezi et al. 2020			*CRP*	*SPARCL*	*µg/ml*	*Individual*			*Median (IQR) / Range*			
		K. pneumoniae					Clinical	18	1.6 (0.5-4.4) / 0.1-8.0			
		E. coli						24	2.0 (0.8-4.8) / 0.1-7.8			
		S. aureus						15	0.8 (0.1-2.0) / 0.0-9.2			
		Strep. spp.						16	0.6 (0.3-1.4) / 0.1-5.4			
		Mycoplasma spp.						18	0.6 (0.3-2.1) / 0.1-6.8			
		Enterococcus spp.						18	0.2 (0.1-0.7) / 0.1-2.6			
		NAS						24	0.1 (0.1-0.1) / 0.1-3.5			
Galfi et al. 2016			*IG*	*RID*	*g/L*	*Quarter*			*Mean (SD) / Range*			
							Healthy	76	24.64 (23.56) / 4.78-162.38			
							Subclinical	74	29.35 (24.38) / 4.62-152.24			
		S. aureus						3	12.21 (8.08)			
		Strep. agalactiae						3	22.51 (15.18)			
		Strep. dysgalactiae						2	28.81 (15.88)			
		E. faecium						1	23.19			
		Coryne. spp.						49	31.15 (27.30)			
		NAS						7	34.11 (21.42)			
		Other bacteria						9	24.68 (17.51)			
Chen et al. 2004			*LF*	*ELISA*	*µg/ml*	*Individual*			*Mean (SD)*			
	<100						SCC group 1	50	176.8 (120.3)			
	100-250						SCC group 2	15	466.0 (508.5)			
	250-500						SCC group 3	10	742.1 (374.2)			
Cheng et al. 2008			*LF*	*ELISA*	*µg/ml*	*Individual*			*Mean (SD)*			

Table 4 (continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Assay	Unit	Sample	Sample group	N	value	%Se	%Sp	Cutoff
	0-18						SCC group 0	12	log 1.914 (0.137)			
	18-35						SCC group 1	20	log 2.022 (0.174)			
	35-71						SCC group 2	50	log 1.980 (0.191)			
	71-141						SCC group 3	40	log 2.058 (0.264)			
	141-283						SCC group 4	34	log 2.098 (0.245)			
	283-566						SCC group 5	20	log 2.262 (0.317)			
	566-1,131						SCC group 6	22	log 2.276 (0.303)			
Sobczuk-Szul et al. 2014			*LF*	*ELISA*	*µg/ml*	*ND*			*Mean*			
	<100						SCC group 1	56	149.16			
	101-400						SCC group 2	66	212.69			
	400-1,000						SCC group 3	21	233.20			
	>1,000						SCC group 4	18	246.77			
Galfi et al. 2016			*LF*	*ELISA*	*mg/ml*	*Quarter*			*Mean (SD) / Range*			
							Healthy	76	5.12 (1.77) / 0.73-8.85			
							Subclinical	74	5.94 (1.65) / 2.26-9.84			
		S. aureus						3	6.21 (0.50)			
		Strep. agalactiae						3	6.48 (0.51)			
		Strep. dysgalactiae						2	4.71 (0.38)			
		E. faecium						1	5.88			
		Coryne. spp.						49	6.05 (1.68)			
		NAS						7	5.30 (1.36)			
		Other bacteria						9	5.88 (2.35)			
Galfi et al. 2016			*LF*	*ELISA*	*mg/ml*	*Quarter*			*Mean (SD) / Range*			

Table 4 (continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Assay	Unit	Sample	Sample group	N	value	%Se	%Sp	Cutoff
		Major						10	5.45 (1.69) / 1.17-6.88			
		Contagious						7	5.61 (2.00) / 1.17-6.88			
		S. aureus						4	4.95 (2.55) / 1.17-6.68			
		Strep. agalactiae						3	6.48 (0.50) / 5.9-6.88			
		Environmental						3	5.10 (0.68) / 4.68-5.88			
		Strep. dysgalactiae						2	4.71 (0.04) / 4.68-4.73			
		E. faecium						1	5.88			
		Minor						56	5.95 (1.65) / 2.40-9.84			
		Coryne. spp.						49	6.05 (1.68) / 2.40-9.84			
		NAS						7	5.30 (1.36) / 4.07-7.60			
		Other bacteria						9	5.88 (2.35) / 2.26-8.34			
		Negative					Healthy	76	5.12 (1.77) / 0.73-8.85			
Zeng et al. 2009			*LBP*	*ELISA*	*µg/ml*	*Quarter*			*Mean (SD)*			
	< 250						SCC group 1	347	6.20 (0.33)			
	> 250						SCC group 2	46	12.78 (1.39)			
Wenzet al. 2010			*LBP*	*ELISA*	*µg/ml*	*Quarter*			*Mean (95% CI)*			
							Clinical (mild)	87	33.3 (25.1-44.3)			
							Clinical (moderate-severe)	60	60.9 (43.8-84.8)			
		Gram-negative						83	51.0 (38.0-68.5)			
		Gram-positive						64	35.2 (25.6-48.4)			
		NAS						19	42.4 (24.7-73.0)			
		Strep. spp.						45	32.6 (22.4-47.3)			
		E. coli						57	50.7 (36.3-70.8)			
		Coliforms						26	51.5 (31.2-85.0)			

Table 5 Cow, results obtained for cytokine markers by ELISA.

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Unit	Sample	Sample group	N	Value	%Se	%Sp	Cutoff
Wenz et al. 2010			*IL-10*	*U/mL*	*Quarter*			*Mean (95% CI)*			
						Clinical (mild)	87	16.9 (11.3-25.5)			
						Clinical (moderate-severe)	60	48.9 (30.1-79.5)			
						Gram-negative	83	48.7 (31.9-74.2)			
						Gram-positive	64	16.3 (10.3-25.8)			
		NAS					19	7.60 (3.55-16.3)			
		Strep. spp.					45	21.8 (12.9-36.9)			
		E. coli					57	53.6 (33.6-85.6)			
		Coliforms					26	39.0 (19.3-78.8)			
Wenz et al. 2010			*IL-12*	*U/mL*	*Quarter*			*Mean (95% CI)*			
						Clinical (mild)	87	153 (102-229)			
						Clinical (moderate-severe)	60	339 (213-539)			
						Gram-negative	83	210 (138-320)			
						Gram-positive	64	270 (172-426)			
		NAS					19	186 (86-402)			
		Strep. spp.					45	313 (185-532)			
		E. coli					57	218 (136-350)			
		Coliforms					26	195 (96-396)			
Wenz et al. 2010			*IL-1β*	*ng/mL*	*Quarter*			*Mean (95% CI)*			
						Clinical (mild)	87	2.59 (2.00-3.35)			
						Clinical (moderate-severe)	60	4.22 (3.15-5.66)			
		Gram-negative					83	4.35 (3.33-5.68)			
		Gram-positive					64	2.55 (1.91-3.41)			
		NAS					19	1.72 (1.06-2.79)			
		Strep. spp.					45	2.99 (2.14-4.17)			
		E. coli					57	4.50 (3.34-6.06)			
		Coliforms					26	4.04 (2.58-6.32)			
Sobczuk-Szul et al. 2014			*IL-1β*	*ng/ml*	*ND*			*Mean*			
	<100					SCC group 1	56	0.08			
	101-400					SCC group 2	66	0.15			
	400-1,000					SCC group 3	21	0.19			
	>1,000					SCC group 4	18	0.21			
Sakemi et al. 2011	*Mean / Range*		*IL-6*	*pg/ml*	*Quarter*			*Mean (SD)*			
	36 / 11-74					SCC Low	37	12.6 (33.4)			
	770 / 130-3,310					SCC High	40	207.0 (441.6)			
Sobczuk-Szul et al. 2014			*IL-6*	*ng/ml*	*ND*			*Mean*			

Table 5 (continued)

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Unit	Sample	Sample group	N	Value	%Se	%Sp	Cutoff
	<100					SCC group 1	56	0.06			
	101-400					SCC group 2	66	0.11			
	400-1,000					SCC group 3	21	0.08			
	>1,000					SCC group 4	18	0.04			
									40	92	80
Wenz et al. 2010			*TGF-α*	*pg/mL*				*Mean*			
					Quarter	Clinical (mild)	87	109			
						Clinical (moderate-severe)	60	168			
		Gram-negative					83	137			
		Gram-positive					64	107			
		NAS					19	91			
		Strep. spp.					45	111			
		E. coli					57	151			
		Coliforms					26	107			
Wenz et al. 2010			*TGF-β1*	*ng/mL*	*Quarter*			*Mean*			
						Clinical (mild)	87	6.80			
						Clinical (moderate-severe)	60	6.61			
		Gram-negative					83	7.20			
		Gram-positive					64	7.08			
		NAS					19	7.66			
		Strep. spp.					45	7.07			
		E. coli					57	6.58			
		Coliforms					26	9.11			
Sobczuk-Szul et al. 2014			*TNF-α*	*ng/ml*	*ND*			*Mean*			
	<100					SCC group 1	56	0.81			
	101-400					SCC group 2	66	0.63			
	400-1,000					SCC group 3	21	0.90			
	>1,000					SCC group 4	18	0.08			

Table 6 Sheep, results obtained for all markers by ELISA.

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Unit	Sample	Sample group	N.	Results	%Se	%Sp	Cutoff
*Winter et al. 2002**			*Cytokines*	*ng/ml*	*Half-udder*			*Mean (SD)*			
			IL-1β			NA	10	NA			
			IL-6			peak at 8h p.i.	10	2.1 (2.1)			
			IL-8			peak at 8h p.i.	10	45.5 (4.1)			
Miglio et al. 2013			*M-SAA*	*μg/mL*	*Half-udder*			*Mean (SD) / Range*			
		NAS, *S. aureus*,				Latent mastitis	11	44.59 (42.07) / 7.77-137.09			
		E. faecalis, S. uberis				Subclinical mastitis	25	114.37 (41.14) / 18.41-142.43			
Addis et al. 2016			*CATH*	*NOD450*	*Half-udder*						
	>500					SCC group 1			86.2	94.6	0.014
	>1,000					SCC group 2			93.4	94.3	0.040
Puggioni et al. 2020	*Median (IQR)*		*CATH*	*AOD450*	*Half-udder*	*Late lactation*		*Median (IQR)*			
	235 (122.5-554.5)	NAS, *E. faecalis*,				Culture negative	281	0.0861 (0.0701-0.1071)	91.2	82.9	0.121
	1,637 (842.8-14,422)	*S. uberis, Klebsiella* spp.				Culture positive	34	0.2261 (0.1352-2.275)			

*Experimental infection study

Table 7 Goat, results obtained for all markers by ELISA.

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Unit	Sample	Sample group	N.	Results	%Se	%Sp	Cutoff
Chen et al. 2004			*LF*	*μg/ml*	*bulk*			*Mean (SD)*			
						MBRT (>8hr)	54	167 (49)			
						MBRT (5-8hr)	47	218 (77)			
						MBRT (<5hr)	5	304 (87)			
						MBRT (4-4.5hr)	10	587 (120)			
						0-72hr p.i.	3	Range: 10-30			
Tedde et al. 2019			*CATH*	*NOD450*	*half-udder*						
							360		85.71	40.41	0.014
Puggioni et al. 2020	*Median (IQR)*		*CATH*	*AOD450*	*half-udder*	*Late lactation*		*Median (IQR)*			
	303 (104-772.5)	NAS, *S. aureus*,				Culture negative	189	0.1121 (0.0886-0.1501)	76.47	57.14	0.118
	812.5 (232.3-2,397)	*S. dysgalactiae*				Culture positive	34	0.1148 (0.1152-0.2449)			

Table 8 Water buffalo, results obtained for all markers by ELISA.

Study	SCC (cells/ml x 10^3)	Pathogens	Marker	Unit	Sample	Sample group	N	Value	%Se	%Sp	Cutoff
Ozenc et al. 2019	*Range*		*LF*	*log*	*Quarter*			*Mean (SD)*			
	0-18					SCC group 0	194	1.22 (0.21)			
	18-36					SCC group 1	77	1.46 (0.30)			
	36-71					SCC group 2	63	1.43 (0.26)			
	71-142					SCC group 3	57	1.51 (0.25)			
	0-18					SCC group 0	7	1.51 (0.33)			
	18-36					SCC group 1	7	1.67 (0.17)			
	36-71					SCC group 2	9	1.73 (0.38)			
	71-142					SCC group 3	12	1.68 (0.36)			
	142-283					SCC group 4	17	1.82 (0.43)			
	283-566					SCC group 5	11	1.91 (0.53)			
	566-1,132					SCC group 6	9	2.01 (0.55)			
	1,132-2,263					SCC group 7	7	2.14 (0.57)			
	2,263-4,536					SCC group 8	5	2.58 (0.40)			
		Total					84	1.85 (0.47)			
		S. aureus					13	2.22 (0.57)			
		NAS					23	1.74 (0.40)			
		Candida spp.					26	1.69 (0.38)			
		S. agalactiae					6	2.25 (0.48)			
		Bacillus spp.					5	1.77 (0.27)			
		E. coli					4	1.64 (0.48)			
		S. aureus/Candida spp.					3	2.08 (0.60)			
		S. aureus/NAS					2	2.20 (0.08)			
		E. coli/NAS					2	1.57 (0.18)			
									97.62	25.83	15.01
									91.67	61.89	25.4
									82.14	78.77	32.9
									47.62	92.07	50.3
									29.76	97.70	101.7
Puggioni et al. 2020	*Median (IQR)*		*CATH*	*AOD450*	*Quarter*			*Median (IQR)*			
	222 (64-985)					Subclinical mastitis	235	0.120 (0.100–0.189)			
	4,091 (2,789-5,173)					Clinical mastitis	7	0.306 (0.133-0.485)			
	324 (103.5–1,556)	*S. aureus*					221	0.112 (0.094–0.138)			
	105 (46–473.5)	NAS					137	0.102 (0.092-0.122)			
	77 (37.2-348)	Other					126	0.110 (0.090-0.131)			
	90 (45-382)	Sterile					63	0.103 (0.090-0.118)			
	259 (59.5-721)	*Strep.* spp./*Ent.* spp.					49	0.105 (0.080-0.1305)			
	199.5 (62.5-667.3)	*Gram-negatives*					22	0.110 (0.090-0.131)			
									57.85	84.09	

Conclusions and recommendations

Our work aimed at analytically assessing the scientific literature describing the use of non-enzymatic milk proteins as mastitis markers in dairy ruminant species with the PRISMA approach. We aimed to summarize and compare the diagnostic performances of the immunoassays developed for their detection in the milk. As expected, the most frequently mentioned biomarkers were M-SAA, HP, CATH, and LF, which were investigated in experimental/observational studies and discovery/implementation approaches. We observed several critical issues in study designs, reference standard methods (the lack of "gold standard"), index tests (frequently performed without a blind approach), heterogeneity in the unit of measurement used for detecting the same biomarker, and the different type of statistical analysis performed, resulting in a heterogeneity of the collected data that was not amenable to meta-analysis. This is a common finding in many meta-analyses and illustrates how important it is for case definitions and other criteria to be standardized between studies. Being related to the nature of the disease, some of these issues could not be solved, because a truly reliable, sensitive, and specific reference diagnostic test does not exist. To deal with this, we applied an alternative synthesis method newly used in systematic reviews, the *"Synthesis Without Meta-analysis"* (SWiM), which improves transparency in reporting. The critical issues we observed further highlight the importance of title writing and keyword definition in the publishing and searching phases. When drafting these crucial parts of their manuscripts, using appropriate consensus terminology will maximize retrieval in bibliographic searches, enhancing article visibility and data usability.

Supplementary Information

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Conflicts of interest/Competing interests

The authors declare no competing interests

Availability of data and material

The data used for the systematic review are presented in supplementary tables.

Authors' contributions

AG: conceptualization, data analysis, data editing, manuscript drafting. MP: data analysis, manuscript drafting. ST: data analysis, manuscript drafting. SD: conceptualization, data analysis, data editing, manuscript drafting, and revision. MFA: conceptualization, data analysis, data editing, manuscript drafting, and revision.

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References

Addis MF, Bronzo V, Puggioni GMG, Cacciotto C, Tedde V, Pagnozzi D, Locatelli C, Casula A, Curone G, Uzzau S, Moroni P (2017) Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis. J Dairy Sci 100:2944–2953. https://doi.org/10.3168/jds.2016-12110

Addis MF, Pisanu S, Marogna G, Cubeddu T, Pagnozzi D, Cacciotto C, Campesi F, Schianchi G, Rocca S, Uzzau S (2013) Production and Release of Antimicrobial and Immune Defense Proteins by Mammary Epithelial Cells following Streptococcus uberis Infection of Sheep. Infect Immun 81:3182–3197. https://doi.org/10.1128/IAI.00291-13

Addis MF, Tedde V, Dore S, Pisanu S, Puggioni GMG, Roggio AM, Pagnozzi D, Lollai S, Cannas A, Uzzau S (2016a) Evaluation of milk cathelicidin for detection of dairy sheep mastitis. J Dairy Sci 99:6446–6456. https://doi.org/10.3168/jds.2015-10293

Addis MF, Tedde V, Puggioni GMG, Pisanu S, Casula A, Locatelli C, Rota N, Bronzo V, Moroni P, Uzzau S (2016b) Evaluation of milk cathelicidin for detection of bovine mastitis. J Dairy Sci 99:8250–8258. https://doi.org/10.3168/jds.2016-11407

Åkerstedt M, Forsbäck L, Larsen T, Svennersten-Sjaunja K (2011) Nat- ural variation in biomarkers indicating mastitis in healthy cows. J Dairy Res 78:88–96. https://doi.org/10.1017/S0022029910000786

Åkerstedt M, Waller KP, Sternesjö Å (2007) Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples. J Dairy Res 74:198–203. https://doi.org/10.1017/S0022029906002305

Åkerstedt M, Waller KP, Sternesjö Å (2009) Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk quality. J Dairy Res 76:483–489. https://doi.org/10.1017/S0022029909990185

Bergonier D, de Crémoux R, Rupp R, Lagriffoul G, Berthelot X (2003) Mastitis in Dairy Small Ruminants. Vet Res 34:689–716. https:// doi.org/10.1051/vetres:2003030

Bochniarz M, Szczubiał M, Brodzki P, Krakowski L, Dąbrowski R (2020) Serum amyloid A as a marker of cow᾽s mastitis caused by Streptococcus sp. Comp Immunol Microbiol Infect Dis 72:101498. https://doi.org/10.1016/j.cimid.2020.101498

Brenaut P, Lefèvre L, Rau A, Laloë D, Pisoni G, Moroni P, Bevilacqua C, Martin P (2014) Contribution of mammary epithelial cells to the immune response during early stages of a bacterial infection to Staphylococcus aureus. Vet Res 45:16. https://doi.org/10.1186/ 1297-9716-45-16

Campbell M, McKenzie JE, Sowden A, Katikireddi SV, Brennan SE, Ellis S, Hartmann-Boyce J, Ryan R, Shepperd S, Thomas J, Welch V (2020) Thomson H (2020) Synthesis without meta-analysis (SWiM) in systematic reviews: reporting guideline. BMJ 368:l6890. https://doi.org/10.1136/bmj.l6890

Chakraborty S, Dhama K, Tiwari R, Iqbal Yatoo M, Khurana SK, Khandia R, Munjal A, Munuswamy P, Kumar MA, Singh M, Singh R, Gupta VK (2019) Chaicumpa W (2019) Technologi- cal interventions and advances in the diagnosis of intramammary infections in animals with emphasis on bovine population—a review. Vet Q 39(1):76–94. https://doi.org/10.1080/01652176. 2019.1642546

Chen PW, Chen WC, Mao FC (2004) Increase of lactoferrin concentration in mastitic goat milk. J Vet Med Sci 66:345–350. https://doi. org/10.1292/jvms.66.345

Chen PW, Mao FC (2004) Detection of lactoferrin in bovine and goat milk by enzyme-linked immunosorbent assay. J Food Drug Anal 12:133–139. https://doi.org/10.38212/2224-6614.2653

Cheng JB, Wang JQ, Bu DP, Liu GL, Zhang CG, Wei HY, Zhou LY, Wang JZ (2008) Factors affecting the lactoferrin concentration in bovine milk. J Dairy Sci 91:970–976. https://doi.org/10.3168/ jds.2007-0689

Contreras A, Sierra D, Sánchez A, Corrales JC, Marco JC, Paape MJ, Gonzalo C (2007) Mastitis in small ruminants. Small Rum Res 68:145–153. https://doi.org/10.1016/j.smallrumres.2006.09.011

Cubeddu T, Cacciotto C, Pisanu S, Tedde V, Alberti A, Pittau M, Dore S, Cannas A, Uzzau S, Rocca S, Addis MF (2017) Cathelicidin production and release by mammary epithelial cells during infectious mastitis. Vet Immunol Immunopathol 189:66–70. https:// doi.org/10.1016/j.vetimm.2017.06.002

Dalanezi FM, Schmidt EMS, Joaquim SF, Guimarães FF, Guerra ST, Lopes BC, Cerri RLA, Chadwick C, Langoni H (2020) Concentrations of Acute-Phase Proteins in Milk from Cows with Clinical Mastitis Caused by Different Pathogens. Pathogens 9:706. https:// doi.org/10.3390/pathogens9090706

Eckersall PD, Young FJ, Nolan AM, Knight CH, McComb C, Waterston MM, Hogarth CJ, Scott EM, Fitzpatrick JL (2006) Acute phase proteins in bovine milk in an experimental model of Staphylococcus aureus subclinical mastitis. J Dairy Sci 89:1488– 1501. https://doi.org/10.3168/jds.S0022-0302(06)72216-0

Ezzat Alnakip M, Quintela-Baluja M, Böhme K, Fernández-No I, Caamaño-Antelo S, Calo-Mata P, Barros-Velázquez J (2014) The immunology of mammary gland of dairy ruminants between healthy and inflammatory conditions. J Vet Med 2014:659801. https://doi.org/10.1155/2014/659801

Fetherston CM, Wells JI, Hartmann PE (2006) Severity of mastitis symptoms as a predictor of C-reactive protein in milk and blood during lactation. Breastfeeding Med 1:127–135. https://doi.org/ 10.1089/bfm.2006.1.127

Galfi AL, Radinović MZ, Boboš SF, Pajić MJ, Savić SS, Milanov DS (2016a) Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings. Vet Arhiv 86:487–497

Galfi AL, Radinović MZ, Milanov DS, Savić SS, Boboš SF, Pajić MJ (2016b) Lactoferrin and immunoglobulin G concentration in bovine milk from cows with subclinical mastitis during the late lactation period. Acta Sci Vet 44:6. https://doi.org/10.22456/ 1679-9216.81097

Gerardi G, Bernardini D, Elia CA, Ferrari V, Iob L, Segato S (2009) Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows. J Dairy Res 76:411–417. https://doi.org/10.1017/S0022029909990057

Grönlund U, Hallén Sandgren C, Persson Waller K (2005) Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis. Vet Res 36:191–198. https://doi. org/10.1051/vetres:2004063

Grönlund U, Hultén C, Eckersall PD, Hogarth C, Persson Waller K (2003) Haptoglobin and serum amyloid A in milk and serum during acute and chronic experimentally induced Staphylococcus aureus mastitis. J Dairy Res 70:379–386. https://doi.org/10.1017/s0022029903006484

Gulbe G, Pilmane M, Saulīte V, Doniņa S, Jermolajevs J, Peškova L, Valdovskaet A (2020) Cells and cytokines in milk of sub- clinically infected bovine mammary glands after the use of immunomodulatory composition GLP 810. Mediators Inflamm 2020:8238029. https://doi.org/10.1155/2020/8238029

Hiss S, Mielenz M, Bruckmaier RM, Sauerwein H (2004) Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression. J Dairy Sci 87:3778–3784. https://doi.org/10.3168/jds.S0022- 0302(04)73516-X

Hiss S, Mueller U, Neu-Zahren A, Sauerwein H (2007) Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters. Veterinarni Medicina 52:245–252. https://doi.org/10.17221/1879-VETMED

Hughes K, Watson CJ (2018) The mammary microenvironment in mastitis in humans, dairy ruminants, rabbits and rodents: a one health focus. J Mammary Gland Biol Neoplasia 23:27–41. https://doi.org/10.1007/s10911-018-9395-1

Hussein HA, El-Razik KAEA, Gomaa AM, Elbayoumy MK, Abdelrahman KA, Hosein HI (2018) Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle. Vet World 11:34– 41. https://doi.org/10.14202/vetworld.2018.34-41

Iliev PT, Georgieva TM (2019) Acute phase biomarkers of diseases in small ruminants: an overview. Bulg J Vet Med 22:1–12. https://doi.org/10.15547/10.15547/bjvm.1051

Jaeger S, Virchow F, Torgerson PR, Bischoff M, Biner B, Hartnack S, Rüegg SR (2017) Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis. J Dairy Sci 100:7419–7426. https://doi.org/10.3168/jds.2016-12446

Kelly AL, Leitner G, Merin U (2018) Milk Quality and Udder Health: Test Methods and Standards. Reference Module in Food Science, Elsevier, 2018, ISBN 9780081005965, doi: https://doi.org/10.1016/B978-0-08-100596-5.00951-3.

Kościuczuk EM, Lisowski P, Jarczak J, Strzałkowska N, Jóźwik A, Horbańczuk J, Krzyżewski J, Zwierzchowski L, Bagnicka E (2012) Cathelicidins: family of antimicrobial peptides. A review. Mol Biol Rep 39:10957–10970. https://doi.org/10.1007/ s11033-012-1997-x

Kováč G, Popelková M, Tkáčiková Ľ, Burdová O, Ihnát O (2007) Interrelationship between somatic cell count and acute phase proteins in serum and milk of dairy cows. Acta Vet Brno 76:51–57. https://doi.org/10.2754/avb200776010051

Kovačević-Filipović M, Ilić V, Vujčić Z, Dojnov B, Stevanov-Pavlović M, Mijačević Z, Božić T (2012) Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis. Vet Immunol Immunopathol 145:120–128. https://doi.org/10.1016/j.vetimm.2011.10.015

Lam TJGM, Olde Riekerink RGM, Sampimon OC, Smith H (2009) Mastitis diagnostics and performance monitoring: a practical approach. Ir Vet J 62:S34–S39. https://doi.org/10.1186/2046-0481-62-S4-S34

Larson MA, Weber A, Weber AT, McDonald TL (2005) Differential expression and secretion of bovine serum amyloid A3 (SAA3) by mammary epithelial cells stimulated with prolactin or lipopolysac- charide. Vet Immunol Immunopathol 107:255–264. https://doi.org/10.1016/j.vetimm.2005.05.006

Lash JA, Coates TD, Lafuze J, Baehner RL, Boxer LA (1983) Plasma lactoferrin reflects granulocyte activation in vivo. Blood 61:885– 888. https://doi.org/10.1182/blood.V61.5.885.885

Leitner G, Shoshani E, Krifucks O, Chaffer M, Saran A (2000) Milk leucocyte population patterns in bovine udder infection of different aetiology. J Vet Med B Infect Dis Vet Public Health 47:581– 589. https://doi.org/10.1046/j.1439-0450.2000.00388.x

Lopez-Benavides MG, Dohoo I, Scholl D, Middleton J, Perez R (2012) Interpreting Bacteriological Culture Results to Diagnose Bovine Intramammary Infections. National Mastitis Council Research Committee Report

McDonald TL, Larson MA, Mack DR, Weber A (2001) Elevated extrahepatic expression and secretion of mammary-associated serum amyloid A 3 (M-SAA3) into colostrum. Vet Immunol Immunopathol 83:203–211. https://doi.org/10.1016/S0165-2427(01)00380-4

Menzies PI, Ramanoon SZ (2001) Mastitis of Sheep and Goats. Vet Clin North Am Food Anim Pract 17:333–358. https://doi.org/10. 1016/S0749-0720(15)30032-3

Miglio A, Moscati L, Fruganti G, Pela M, Scoccia E, Valiani A, Maresca C (2013) Use of milk amyloid A in the diagnosis of sub- clinical mastitis in dairy ewes. J Dairy Res 80:496–502. https:// doi.org/10.1017/S0022029913000484

Moher D, Liberati A, Tetzlaff J, Altman DG (2009) Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement. PLoS Med 6:e1000097. https://doi.org/10.1371/journ al.pmed.1000097

Oliver SP, Gonzales RN, Hogan JS, Jayarao BM, Owens WE, Owens W (2004). Microbiological procedures for the diagnosis of bovine udder infection and determination of milk quality. 4th Edition. National Mastitis Council, Inc. Verona (WI), USA.

Özenç E, Şeker E, Acar D, Koca HB, Yazıcı E, Çelik H, Doğan N, Avcı G, Yılmaz O, Küçükkebapçı M, Uçar M, Baştan A (2019) Milk lactoferrin concentrations in anatolian buffaloes with and without subclinical mastitis. Buffalo Bull 38:2941–2298

Pedersen LH, Aalbaek B, Røntved CM, Ingvartsen KL, Sorensen NS, Heegaard PM, Jensen HE (2003) Early pathogenesis and inflammatory response in experimental bovine mastitis due to Streptococcus uberis. J Comp Pathol 128:156–164. https://doi.org/10. 1053/jcpa.2002.0620

Persson Y, Olofsson I (2011) Direct and indirect measurement of somatic cell count as indicator of intramammary infection in dairy goats. Acta Vet Scand 53:15. https://doi.org/10.1186/ 1751-0147-53-15

Pisanu S, Cacciotto C, Pagnozzi D, Uzzau S, Pollera C, Penati M, Bronzo V, Addis MF (2020) Impact of Staphylococcus aureus infection on the late lactation goat milk proteome: new perspectives for monitoring and understanding mastitis in dairy goats. J Proteomics 221:103763. https://doi.org/10.1016/j.jprot.2020. 10376

Puggioni GMG, Tedde V, Uzzau S, Dore S, Liciardi M, Cannas EA, Pollera C, Moroni P, Bronzo V, Addis MF (2020b) Relationship of late lactation milk somatic cell count and cathelicidin with intramammary infection in small ruminants. Pathogens 9:37. https://doi.org/10.3390/pathogens9010037

Puggioni GMG, Tedde V, Uzzau S, Guccione J, Ciaramella P, Pollera C, Moroni P, Bronzo V, Addis MF (2020a) Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: comparison with milk somatic cell count and bacteriological culture. Res Vet Sci 128:129–134. https://doi.org/10.1016/j.rvsc. 2019.11.009

Pyörälä S, Hovinen M, Simojoki H, Fitzpatrick J, Eckersall PD, Orro T (2011) Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens. Vet Rec 168:535–535. https://doi.org/10.1136/vr.d1120

Rossi RS, Amarante AF, Correia LBN, Guerra ST, Nobrega DB, Latosinski GS, Rossi BF, Rall VLM, Pantoja JCF (2018) Diagnostic accuracy of Somaticell, California Mastitis Test, and microbiological examination of composite milk to detect Streptococcus agalactiae intramammary infections. J Dairy Sci 101:10220– 10229. https://doi.org/10.3168/jds.2018-14753

Ruegg PL (2017) A 100-Year Review: Mastitis detection, management, and prevention. J Dairy Sci 100:10381–10397. https://doi.org/10. 3168/jds.2017-13023

Safi S, Khoshvaghti A, Jafarzadeh SR, Bolourchi M, Nowrouzian I (2009) Acute phase proteins in the diagnosis of bovine subclinical mastitis. Vet Clin Pathol 38:471–476. https://doi.org/10.1111/j. 1939-165X.2009.00156.x

Sakemi Y, Tamura Y, Hagiwara K (2011) Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis. J Dairy Res 78:118–121. https://doi.org/10.1017/S0022029910000828

Sargeant JM, O'Connor AM (2020) Scoping Reviews, Systematic Reviews, and Meta-Analysis: Applications in Veterinary Medi- cine. Front Vet Sci 7:11. https://doi.org/10.3389/fvets.2020.00011

Shaheen T, Bilal Ahmad S, Rehman MU, Muzamil S, Bhat RR, Hussain I, Bashir N, Mir MUR, Paray BA, Dawood MAO (2020) Investigations on cytokines and proteins in lactating cows with and without naturally occurring mastitis. Journal of King Saud University - Science 32:2863–2867. https://doi.org/10.1016/j. jksus.2020.07.009

Shimazaki K, Kawai K (2017) Advances in lactoferrin research con- cerning bovine mastitis. Biochem Cell Biol 95:69–75. https://doi. org/10.1139/bcb-2016-0044

Shirazi-Beheshtiha SH, Safi S, Rabbani V, Bolourchi M, Ameri M, Khansari MR (2011) The diagnostic value of determination of positive and negative acute phase proteins in milk from dairy cows with subclinical mastitis. Comp Clin Pathol 21:999–1003. https:// doi.org/10.1007/s00580-011-1216-5

Smolenski GA, Wieliczko RJ, Pryor SM et al (2011) The abundance of milk cathelicidin proteins during bovine mastitis. Vet Immunol Immunopathol 143:125–130. https://doi.org/10.1016/j.vetimm. 2011.06.034

Sobczuk-Szul M, Wielgosz-Groth Z, Nogalski Z, Pogorzelska- Przybyłek P (2014) Changes in the content of whey proteins dur- ing lactation in cow's milk with a different somatic cells count. Vet Med Zoot 65(87)

Sordillo LM, Streicher KL (2002) Mammary gland immunity and mas- titis susceptibility. J Mammary Gland Biol Neoplasia 7:135–146. https://doi.org/10.1023/a:1020347818725

Souza FN, Blagitz MG, Penna CFAM, Della Libera AMMP, Heinemann MB, Cerqueira MMOP (2012) Somatic cell count in small ruminants: Friend or foe? Small Rum Res 107:65–75. https://doi. org/10.1016/j.smallrumres.2012.04.005

Suojala L, Orro T, Järvinen H, Saatsi J, Pyörälä S (2008) Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows. Acta Vet Scand 50(18). https://doi.org/10.1186/1751-0147-50-18

Szczubiał M, Dąbrowski R, Kankofer M, Bochniarz M, Komar M (2012) Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens. Pol J Vet Sci 15:291–296. https://doi.org/10. 2478/v10181-011-0149-x

Tedde V, Bronzo V, Puggioni GMG, Pollera C, Casula A, Curone G, Moroni P, Uzzau S, Addis MF (2019) Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation. J Dairy Res 86:217–221. https://doi.org/10.1017/S002202991 9000335

Thomas FC, Waterston M, Hastie P, Parkin T, Haining H, Eckersall PD (2015) The major acute phase proteins of bovine milk in a commercial dairy herd. BMC Vet Res 11:207. https://doi.org/10. 1186/s12917-015-0533-3

Tuaillon E, Viljoen J, Dujols P, Cambonie G, Rubbo PA, Nagot N, Bland RM, Badiou S, Newell ML, Van de Perre P (2017) Subclinical mastitis occurs frequently in association with dramatic changes in inflammatory/anti-inflammatory breast milk components. Pediatr Res 81:556–564. https://doi.org/10. 1038/pr.2016.220

Van Harten RM, van Woudenbergh E, van Dijk A, Haagsman HP (2018) Cathelicidins: Immunomodulatory Antimicrobials. Vaccine (Basel) 6:63. https://doi.org/10.3390/vaccines60 30063

Viguier C, Arora S, Gilmartin N, Welbeck K, O'Kennedy R (2009) Mastitis detection: current trends and future perspectives. Trends in Biotechnol 27:486–493. https://doi.org/10.1016/j.tibtech.2009. 05.004

Welty FK, Larry Smith K, Schanbacher FL (1976) Lactoferrin Concentration During Involution of the Bovine Mammary Gland. J Dairy Sci 59:224–231. https://doi.org/10.3168/jds.S0022-0302(76) 84188-4

Wenz JR, Fox LK, Muller FJ, Rinaldi M, Zeng R, Bannerman DD (2010) Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis. J Dairy Sci 93:2458–2470. https://doi.org/10. 3168/jds.2009-2819

Winter P, Colditz IG (2002) Immunological responses of the lactating ovine udder following experimental challenge with Staphylococcus epidermidis. Vet Immunol Immunopathol 89:57–65. https:// doi.org/10.1016/s0165-2427(02)00184-8

Wollowski L, Heuwieser W, Kossatz A, Addis MF, Puggioni GMG, Meriaux L, Bertulat S (2021) The value of the biomarkers cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis. J Dairy Sci 104:2106–2122. https://doi.org/10.3168/jds.2020-18539

Zanetti M (2004) Cathelicidins, multifunctional peptides of the innate immunity. J Leukocyte Biol 75:39–48. https://doi.org/10.1189/ jlb.0403147

Zanetti M (2005) The role of cathelicidins in the innate host defenses of mammals. Curr Issues Mol Biol 7:179–196

Zeng R, Bequette BJ, Vinyard BT, Bannerman DD (2009) Determination of milk and blood concentrations of lipopolysaccharide- binding protein in cows with naturally acquired subclinical and clinical mastitis. J Dairy Sci 92:980–989. https://doi.org/10.3168/ jds.2008-1636

Appendix

Supplementary tables

Table I. Organization of the keywords by category and combinations used for the database searches

	Object (OR)	**Matrix**	**Diagnosis (OR)**	**Assay (OR)**
KW	*- biomarker - marker - amyloid - haptoglobin - cathelicidin - lactoferrin*	*milk*	*- "intramammary infection" - Mastitis*	*- immunoassay - ELISA - "lateral flow" - immunochromatography*
I.	Marker	milk	mastitis	immunoassay
II.	Biomarker	milk	mastitis	immunoassay
III.	Amyloid	milk	mastitis	immunoassay
IV.	Haptoglobin	milk	mastitis	immunoassay
V.	Cathelicidin	milk	mastitis	immunoassay
VI.	Lactoferrin	milk	mastitis	immunoassay
VII.	Marker	milk	mastitis	ELISA
VIII.	Biomarker	milk	mastitis	ELISA
IX.	Amyloid	milk	mastitis	ELISA
X.	Haptoglobin	milk	mastitis	ELISA
XI.	Cathelicidin	milk	mastitis	ELISA
XII.	Lactoferrin	milk	mastitis	ELISA
XIII.	Marker	milk	mastitis	"lateral flow"
XIV.	Biomarker	milk	mastitis	"lateral flow"
XV.	Amyloid	milk	mastitis	"lateral flow"
XVI.	Haptoglobin	milk	mastitis	"lateral flow"
XVII.	Cathelicidin	milk	mastitis	"lateral flow"
XVIII.	Lactoferrin	milk	mastitis	"lateral flow"
XIX.	Marker	milk	mastitis	immunochromatography
XX.	Biomarker	milk	mastitis	immunochromatography
XXI.	Amyloid	milk	mastitis	immunochromatography
XXII.	Haptoglobin	milk	mastitis	immunochromatography
XXIII.	Cathelicidin	milk	mastitis	immunochromatography
XXIV.	Lactoferrin	milk	mastitis	immunochromatography
XXV.	Marker	milk	"intramammary infection"	immunoassay
XXVI.	Biomarker	milk	"intramammary infection"	immunoassay
XXVII.	Amyloid	milk	"intramammary infection"	immunoassay
XVIII.	Haptoglobin	milk	"intramammary infection"	immunoassay
XXIX.	Cathelicidin	milk	"intramammary infection"	immunoassay
XXX.	Lactoferrin	milk	"intramammary infection"	immunoassay
XXXI.	Marker	milk	"intramammary infection"	ELISA
XXXII.	Biomarker	milk	"intramammary infection"	ELISA
XXIII.	Amyloid	milk	"intramammary infection"	ELISA
XXIV.	Haptoglobin	milk	"intramammary infection"	ELISA
XXXV.	Cathelicidin	milk	"intramammary infection"	ELISA
XXVI.	Lactoferrin	milk	"intramammary infection"	ELISA
XXVII.	Marker	milk	"intramammary infection"	"lateral flow"
XVIII.	Biomarker	milk	"intramammary infection"	"lateral flow"
XXIX.	Amyloid	milk	"intramammary infection"	"lateral flow"
XL.	Haptoglobin	milk	"intramammary infection"	"lateral flow"
XLI.	Cathelicidin	milk	"intramammary infection"	"lateral flow"
XLII.	Lactoferrin	milk	"intramammary infection"	"lateral flow"

Table II. MedLine search results

Title	Authors/Year	DOI
[C-reactive protein as a new parameter of mastitis]	Schrödl W et al 1995	
15-F2t-Isoprostane Concentrations and Oxidant Status in Lactating Dairy Cattle with Acute Coliform Mastitis	Mavangira V et al 2016	10.1111/jvim.13793
A pilot study of acute phase proteins as indicators of bovine mastitis caused by different pathogens	Thomas FC et al. 2018	10.1016/j.rvsc.2018.06.015
A proteomic perspective on the changes in milk proteins due to high somatic cell count	Zhang L et al. 2015	10.3168/jds.2014-9279
A proteomics-based identification of putative biomarkers for disease in bovine milk	van Altena SE et al. 2016	10.1016/j.vetimm.2016.04.005
Advances in BHV1(IBR) research	Straub OC. 2001	
Association of polymorphism within LTF gene promoter with lactoferrin concentration in milk of Holstein cows	Zabolewicz T et al. 2014	10.2478/pjvs-2014-0094
Biosensor assay for determination of haptoglobin in bovine milk	Åkerstedt M et al. 2006	10.1017/S0022029906001774
Bovine intra-mammary challenge with Streptococcus dysgalactiae spp. Dysgalactiae to explore the effect on the response of Complement activity	Maye S et al. 2017	10.1017/S0022029917000292
Cathelicidin production and release by mammary epithelial cells during infectious mastitis	Cubeddu T et al. 2017	10.1016/j.vetimm.2017.06.002
Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens	Szczubiał M et al. 2012	10.2478/v10181-011-0149-x
Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis	Zeng R et al. 2009	10.3168/jds.2008-1636
Development of an immunosensor assay for detection of haptoglobin in mastitic milk	Tan X et al. 2012	10.1111/j.1939-165X.2012.00468.x
Dynamics of experimentally induced Staphylococcus epidermidis mastitis in East Friesian milk ewes	Winter P et al. 2003	10.1017/s002202990300606x
Early pathogenesis and inflammatory response in experimental bovine mastitis due to Streptococcus uberis	Pedersen LH et al. 2003	10.1053/jcpa.2002.0620
Early post parturient changes in milk acute phase proteins	Thomas FC et al. 2016	10.1017/S0022029916000297
Effect of intramammary infusion of tumour necrosis factor-alpha on milk protein composition and induction of acute-phase protein in the lactating cow	Watanabe A et al. 2000	10.1046/j.1439-0450.2000.00400.x
Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro	Danowski K et al. 2013	10.1111/j.1439-0396.2012.01305.x
Effects of lactoferrin and milk on adherence of Streptococcus uberis to bovine mammary epithelial cells	Fang W et al. 2000	10.2460/ajvr.2000.61.275
Elevated milk soluble CD14 in bovine mammary glands challenged with Escherichia coli lipopolysaccharide	Lee JW et al. 2003	10.3168/jds.S0022-0302(03)73832-6
Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection	Bannerman DD et al. 2004	10.1128/CDLI.11.3.463-472.2004
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture	Puggioni GMG et al. 2020	10.1016/j.rvsc.2019.11.009
Evaluation of milk cathelicidin for detection of bovine mastitis	Addis MF et al. 2016	10.3168/jds.2016-11407
Evaluation of milk cathelicidin for detection of dairy sheep mastitis	Addis MF et al. 2016	10.3168/jds.2015-10293
Expression of cathelicidins mRNA in the goat mammary gland and effect of the intramammary infusion of lipopolysaccharide on milk cathelicidin-2 concentration	Zhang GW et al. 2014	10.1016/j.vetmic.2014.01.029
Expression of the peptidoglycan recognition protein, PGRP, in the lactating mammary gland	Kappeler SR et al. 2004	10.3168/jds.S0022-0302(04)73392-5
Facile construction of a molecularly imprinted polymer-based electrochemical sensor for the detection of milk amyloid A	Zhang Z et al. 2020	10.1007/s00604-020-04619-7
Factors affecting the lactoferrin concentration in bovine milk	Cheng JB et al. 2008	10.3168/jds.2007-0689
Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis	Wenz JR et al. 2010	10.3168/jds.2009-2819
Generation of an anti-NAGase single chain antibody and its application in a biosensor-based assay for the detection of NAGase in milk	Welbeck K et al, 2011	10.1016/j.jim.2010.09.019
Genetic variability of lactoferrin content estimated by mid-infrared spectrometry in bovine milk	Soyeurt H et al. 2007	10.3168/jds.2006-827
Gold Nanoparticle Size-Dependent Enhanced Chemiluminescence for Ultra-Sensitive Haptoglobin Biomarker Detection	Nirala NR et al. 2019	10.3390/biom9080372
Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression	Hiss S et al. 2004	10.3168/jds.S0022-0302(04)73516-X

Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis	Fang W et al. 1999	10.1111/j.1574-6968.1999.tb13647.x
Immune-associated traits measured in milk of Holstein-Friesian cows as proxies for blood serum measurements	Denholm SJ et al. 2018	10.3168/jds.2018-14825
Immunosensing system for rapid multiplex detection of mastitis-causing pathogens in milk	Juronen D et al. 2018	10.1016/j.talanta.2017.10.043
Increase in milk metalloproteinase activity and vascular permeability in bovine endotoxin-induced and naturally occurring Escherichia coli mastitis	Raulo SM et al. 2002	10.1016/s0165-2427(01)00423-8
Increase of lactoferrin concentration in mastitic goat milk	Chen PW et al. 2004	10.1292/jvms.66.345
Increased Epstein-Barr virus in breast milk occurs with subclinical mastitis and HIV shedding	Sanosyan A et al. 2016	10.1097/MD.0000000000004005
Influence of bacterial factors on proliferation of bovine mammary epithelial cells	Calvinho LF et al. 2001	
Innate immune response in experimentally induced bovine intramammary infection with Staphylococcus simulans and S. epidermidis	Simojoki H et al. 2011	10.1186/1297-9716-42-49
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis	Sakemi Yet al. 2011	10.1017/S0022029910000828
Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with Staphylococcus aureus alpha-toxin	Riollet C et al. 2000	10.1007/s000110050621
Kinetics of local and systemic isoforms of serum amyloid A in bovine mastitic milk	Jacobsen S et al. 2005	10.1016/j.vetimm.2004.09.031
Lactoferrin concentrations in bovine milk prior to dry-off	Newman KA et al. 2009	10.1017/S0022029909990033
Low-level laser therapy attenuates LPS-induced rats mastitis by inhibiting polymorphonuclear neutrophil adhesion	Wang Y et al. 2014	10.1292/jvms.14-0061
Mastitis detection: current trends and future perspectives	Viguier C et al. 2009	10.1016/j.tibtech.2009.05.004
Mastitis is associated with IL-6 levels and milk fat globule size in breast milk	Mizuno K et al. 2012	10.1177/0890334412455946
Mid-infrared prediction of lactoferrin content in bovine milk: potential indicator of mastitis	Soyeurt H et al. 2012	10.1017/S1751731112000791
Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation	Tedde V et al. 2019	10.1017/S0022029919000335
Milk cytokines and subclinical breast inflammation in Tanzanian women: effects of dietary red palm oil or sunflower oil supplementation	Filteau SM et al. 1999	10.1046/j.1365-2567.1999.00834.x
Milk haptoglobin detection based on enhanced chemiluminescence of gold nanoparticles	Nirala NR et al. 2019	10.1016/j.talanta.2019.01.027
Milk prostaglandins and electrical conductivity in bovine mastitis	Atroshi F et al. 1987	10.1007/BF00361322
Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification	Boehmer JL et al. 2010	10.3168/jds.2009-2526
Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection	Addis MF et al. 2011	10.1128/IAI.00040-11
Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis	Addis MF et al. 2017	10.3168/jds.2016-12110
Relationship between milk lactoferrin and etiological agent in the mastitic bovine mammary gland	Chaneton L et al. 2008	10.3168/jds.2007-0732
Relationship of Late Lactation Milk Somatic Cell Count and Cathelicidin with Intramammary Infection in Small Ruminants	Puggioni GMG et al. 2020	10.3390/pathogens9010037
Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis	Kovačević-Filipović M et al. 2003	10.1016/j.vetimm.2011.10.015
Serum C-reactive protein in dairy herds	Lee WC et al. 2003	
Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with Escherichia coli	Ma JL et al. 2011	10.3168/jds.2011-4167
Susceptibility of sows to experimentally induced Escherichia coli mastitis	Ross RF et al. 1983	
Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis	Jaeger S et al. 2017	10.3168/jds.2016-12446
The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence	Molenaar AJ et al. 2009	10.1080/13547500902730714
The Antisecretory Factor in Plasma and Breast Milk in Breastfeeding Mothers-A Prospective Cohort Study in Sweden	Gustafsson A et al. 2018	10.3390/nu10091227
The Effect of Lipopolysaccharide-Induced Experimental Bovine Mastitis on Clinical Parameters, Inflammatory Markers, and the Metabolome: A Kinetic Approach	Johnzon CF et al. 2018	10.3389/fimmu.2018.01487
The major acute phase proteins of bovine milk in a commercial dairy herd	Thomas FC et al. 2015	10.1186/s12917-015-0533-3
The production and characterization of anti-bovine CD14 monoclonal antibodies	Sohn EJ et al. 2004	10.1051/vetres:2004035

The proteomic advantage: label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis	Boehmer JL et al. 2010	10.1016/j.vetimm.2010.10.004
The relationship between the variants of the bovine MBL2 gene and milk production traits, mastitis, serum MBL-C levels and complement activity	Wang X et al. 2012	10.1016/j.vetimm.2012.06.017
Three novel single-nucleotide polymorphisms of complement component 4 gene(C4A) in Chinese Holstein cattle and their associations with milk performance traits and CH50	Yang Y et al. 2012	10.1016/j.vetimm.2011.11.010
Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes	Miglio A et al. 2013	10.1017/S0022029913000484
Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows	Gerardi G et al. 2009	10.1017/S0022029909990057

Table III. Scopus search results

Title	Authors/Year	DOI
[C-reactive protein as a new parameter of mastitis]	Schrödl W et al. 1995	
15-F2t-Isoprostane Concentrations and Oxidant Status in Lactating Dairy Cattle with Acute Coliform Mastitis	Mavangira, V et al. 2016	10.1111/jvim.13793
A pilot study of acute phase proteins as indicators of bovine mastitis caused by different pathogens	Thomas, F.C et al. 2018	10.1016/j.rvsc.2018.06.015
A proteomic perspective on the changes in milk proteins due to high somatic cell count	Zhang, L et al. 2015	10.3168/jds.2014-9279
A proteomics-based identification of putative biomarkers for disease in bovine milk	van Altena et al. 2016	10.1016/j.vetimm.2016.04.005
Advances in BHV1(IBR) research	Straub, O.C. 2001	
Association of polymorphism within LTF gene promoter with lactoferrin concentration in milk of Holstein cows	Zabolewicz T et al. 2014	10.1016/j.vetimm.2016.04.005
Biomarker based detection of subclinical mastitis by liquid phase blocking elisa	Pranayapradhan et al. 2013	
Biosensor assay for determination of haptoglobin in bovine milk	Åkerstedt M et al. 2006	10.1017/S0022029906001774
Bovine intra-mammary challenge with Streptococcus dysgalactiae spp. Dysgalactiae to explore the effect on the response of Complement activity	Maye, S et al. 2017	10.1016/j.vetimm.2016.04.005
Changes in the content of whey proteins during lactation in cow's milk with a different somatic cells count	Sobczuk-Szul M et al. 2014	
Comparative diagnosis of infectious bacteria in bovine milk	Sedky D et al. 2020	10.17582/journal.jahp/2020/8.4.171.182
Concentration of serum amyloid A and activity of ceruloplasmin in milk from cows with clinical and subclinical mastitis	Szczubiał M et al. 2008	10.2478/v10181-011-0149-x
Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens	Szczubiał M et al. 2012	10.2478/v10181-011-0149-x
Concentrations of acute-phase proteins in milk from cows with clinical mastitis caused by different pathogens	Dalanezi FM et al. 2020	10.3390/pathogens9090706
Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis	Zeng R et al. 2009	10.3168/jds.2008-1636
Development of an immunosensor assay for detection of haptoglobin in mastitic milk	Tan X et al. 2012	10.1111/j.1939-165X.2012.00468.x
Early post parturient changes in milk acute phase proteins	Thomas FC et al. 2016	10.1017/S0022029916000297
Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro	Danowski, K et al. 2013	10.1111/j.1439-0396.2012.01305.x
Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in goats	Purba FY et al. 2020	
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture	Puggioni GMG et al. 2020	10.1016/j.rvsc.2019.11.009
Evaluation of intramammary platelet concentrate efficacy as a subclinical mastitis treatment in dairy cows based on somatic cell count and milk amyloid A levels [Sütçü ıneklerde subklinik mastitis tedavisinde meme ıçi platelet konsantresi etkinliğinin somatik hücre sayımı ve süt amiloid a seviyeleri ıle değerlendirilmesi]	Evkuran DG et al. 2019	
Evaluation of milk cathelicidin for detection of bovine mastitis	Addis MF et al. 2016	10.3168/jds.2016-11407
Evaluation of milk cathelicidin for detection of dairy sheep mastitis	Addis MF et al. 2016	10.3168/jds.2015-10293
Expression of cathelicidins mRNA in the goat mammary gland and effect of the intramammary infusion of lipopolysaccharide on milk cathelicidin-2 concentration	Zhang GW et al. 2014	10.1016/j.vetmic.2014.01.029
Facile construction of a molecularly imprinted polymer–based electrochemical sensor for the detection of milk amyloid A	Zhang Z et al. 2020	10.1016/j.vetmic.2014.01.029
Factors affecting the lactoferrin concentration in bovine milk	Cheng JB et al. 2008	10.3168/jds.2007-0689
Generation of an anti-NAGase single chain antibody and its application in a biosensor-based assay for the detection of NAGase in milk	Welbeck K et al. 2011	10.1016/j.jim.2010.09.019
Gold nanoparticle size-dependent enhanced chemiluminescence for ultra-sensitive haptoglobin biomarker detection	Nirala, NR et al. 2019	10.3390/biom9080372
Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters	Hiss S et al. 2007	
Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression	Hiss S et al. 2004	10.3168/jds.S0022-0302(04)73516-X
Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis	Fang W et al. 1999	10.1111/j.1574-6968.1999.tb13647.x

Immune-associated traits measured in milk of Holstein-Friesian cows as proxies for blood serum measurements	Denholm SJ et al. 2018	10.3168/jds.2018-14825
Immunological responses of the lactating ovine udder following experimental challenge with Staphylococcus epidermidis	Winter P et al. 2002	
Immunosensing system for rapid multiplex detection of mastitis-causing pathogens in milk	Juronen D et al. 2018	10.1016/j.talanta.2017.10.043
Increase of lactoferrin concentration in mastitic goat milk	Chen P et al. 2004	10.1292/jvms.66.345
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis	Sakemi Y et al. 2011	10.1017/S0022029910000828
Interrelationship between somatic cell count and acute phase proteins in serum and milk of dairy cows	Kováč G et al. 2007	
Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with Staphylococcus aureus α-toxin	Riollet C et al. 2000	10.1007/s000110050621
Lactoferrin and IgG levels in ovine milk throughout lactation: Correlation with milk quality parameters	Navarro F et al. 2018	
Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings	Galfi A et al. 2016	
Lactoferrin concentrations in bovine milk prior to dry-off	Newman KA et al. 2009	10.1017/S0022029909990033
Mastitis detection: current trends and future perspectives	Viguier C et al. 2009	10.1016/j.tibtech.2009.05.004
Mid-infrared prediction of lactoferrin content in bovine milk: Potential indicator of mastitis	Soyeurt H et al. 2012	10.1017/S1751731112000791
Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation	Tedde V et al. 2019	10.1017/S0022029919000335
Milk cytokines and subclinical breast inflammation in Tanzanian women: Effects of dietary red palm oil or sunflower oil supplementation	Filteau SM et al. 1999	10.1046/j.1365-2567.1999.00834.x
Omic approaches to a better understanding of mastitis in dairy cows(Book Chapter)	Mudaliar M et al. 2017	
Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification	Boehmer JL. et al 2010	10.3168/jds.2009-2526
Purification of prostaglandin D synthase by ceramic- and size exclusion chromatography	Schlatterer JC et al. 2006	
Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis	Addis MF et al. 2017	10.3168/jds.2016-12110
Relationship of late lactation milk somatic cell count and cathelicidin with intramammary infection in small ruminants	Puggioni GMG et al. 2020	10.3390/pathogens9010037
Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis	Kovacevic-Filipovic M et al. 2012	10.1016/j.vetimm.2011.10.015
Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with Escherichia coli	Ma JL et al. 2011	10.3168/jds.2011-4167
Staphylococcus aureus lipoteichoic acid triggers inflammation in the lactating bovine mammary gland	Rainard P et al. 2008	
Susceptibility of sows to experimentally induced Escherichia coli mastitis.	Ross RF et al. 1983	
Technological interventions and advances in the diagnosis of intramammary infections in animals with emphasis on bovine population—a review	Chakraborty S et al. 2019	10.1080/01652176.2019.1642546
Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis	Jaeger S et al. 2017	10.1016/j.vetimm.2011.10.015
The antisecretory factor in plasma and breast milk in breastfeeding mothers—a prospective cohort study in Sweden	Gustafsson A et al. 2018	10.3390/nu10091227
The diagnostic value of determination of positive and negative acute phase proteins in milk from dairy cows with subclinical mastitis	Shirazi-Beheshtiha SH et al. 2012	
The major acute phase proteins of bovine milk in a commercial dairy herd	Thomas FC et al. 2015	10.1186/s12917-015-0533-3
The proteomic advantage: Label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis	Boehmer JL et al. 2010	10.1016/j.vetimm.2010.10.004
The relationship between lactoferrin gene polymorphism and subclinical mastitis in awassi ewes	Alekish M et al. 2019	
Three novel single-nucleotide polymorphisms of complement component 4 gene(C4A) in Chinese Holstein cattle and their associations with milk performance traits and CH50	Yang Y et al. 2012	10.1016/j.vetimm.2011.11.010
Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes	Miglio A et al. 2013	10.1017/S0022029913000484
Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows	Gerardi G et al. 2009	10.1017/S0022029913000484

Table IV. WoS search results

Title	Authors/Year	DOI
15-F-2t-Isoprostane Concentrations and Oxidant Status in Lactating Dairy Cattle with Acute Coliform Mastitis	Mavangira V et al. 2016	10.1111/jvim.13793
A genome-wide association study for natural antibodies measured in blood of Canadian Holstein cows	de Klerk B et al. 2018	10.1186/s12864-018-5062-6
A proteomics-based identification of putative biomarkers for disease in bovine milk	van Altena SEC et al. 2016	10.1016/j.vetimm.2016.04.005
Acute phase response in lame crossbred dairy cattle	Bagga A et al. 2016	10.14202%2Fvetworld.2016.1204-1208
Advances in BHV1(IBR) research	Straub OC 2001	
Assessment of milk quality trough microbiological an cytometric examination and determination of acute-phase proteins	Medvid V et al. 2011	
Association of polymorphism within LTF gene promoter with lactoferrin concentration in milk of Holstein cows	Zabolewicz T et al. 2014	10.2478/pjvs-2014-0094
Binding of bovine lactoferrin to Streptococcus dysgalactiae subsp dysgalactiae isolated from cows with mastitis	Park HM et al. 2002	10.1111/j.1574-6968.2002.tb11057.x
Biosensor assay for determination of haptoglobin in bovine milk	Åkerstedt M et al. 2006	10.1017/S0022029906001774
Bovine intra-mammary challenge with Streptococcus dysgalactiae spp. Dysgalactiae to explore the effect on the response of Complement activity	Maye S et al. 2017	10.1016/j.vetimm.2016.04.005
Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different, pathogens	Szczubial M et al. 2012	10.2478/v10181-011-0149-x
Crosstalk between coagulation and inflammation in mastitis and metritis in dairy cows	Bobowiec r et al. 2009	10.1556/AVet.57.2009.2.9
Cytokine and acute phase protein gene expression in repeated liver biopsies of dairy cows with a lipopolysaccharide-induced mastitis	Vels L et al. 2009	10.3168/jds.2008-1209
Detection of lactoferrin in bovine and goat milk by enzyme-linked immunosorbent assay	Chen PW et al. 2004	10.38212/2224-6614.2653
Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis	Zeng R et al. 2009	10.3168/jds.2008-1636
Development and validation of an ELISA for the quantification of bovine ITIH4 in serum and milk	Soler L et al. 2019	
Development of an immunosensor assay for detection of haptoglobin in mastitic milk	Tan X et al. 2012	10.1111/j.1939-165X.2012.00468.x
Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation	Moyes KM et al. 2009	10.3168/jds.2009-2170
Effect of gestation length on the levels of five innate defence proteins in human milk	Broadhurst M et al. 2015	10.1016/j.earlhumdev.2014.11.006
Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro	Danowski K et al. 2103	10.1111/j.1439-0396.2012.01305.x
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture	Puggioni GMG et al. 2020	10.1016/j.rvsc.2019.11.009
Evaluation of Intramammary Platelet Concentrate Efficacy as a Subclinical Mastitis Treatment in Dairy Cows Based on Somatic Cell Count and Milk Amyloid A Levels	Evkuran DG et al. 2019	10.9775/kvfd.2018.20982
Evaluation of milk cathelicidin for detection of bovine mastitis	Addis MF et al. 2016	10.3168/jds.2016-11407
Evaluation of milk cathelicidin for detection of dairy sheep mastitis	Addis MF et al. 2016	10.3168/jds.2015-10293
Expression of cathelicidins mRNA in the goat mammary gland and effect of the intramammary infusion of lipopolysaccharide on milk cathelicidin-2 concentration	Zhang GW et al. 2014	10.1016/j.vetmic.2014.01.029
Facile construction of a molecularly imprinted polymer-based electrochemical sensor for the detection of milk amyloid A	Zhang ZR et al. 2020	10.1007/s00604-020-04619-7
Factors affecting the lactoferrin concentration in bovine milk	ChengJB et al. 2008	10.3168/jds.2007-0689
Generation of an anti-NAGase single chain antibody and its application in a biosensor-based assay for the detection of NAGase in milk	Welbeck K et al. 2011	10.1016/j.jim.2010.09.019
Gold Nanoparticle Size-Dependent Enhanced Chemiluminescence for Ultra-Sensitive Haptoglobin Biomarker Detection	Nirala NR et al. 2019	10.3390/biom9080372
Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters	Hiss S et al. 2007	10.17221/1879-vetmed
Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression	Hiss S et al. 2004	10.3390/biom9080372

Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis	Fang et al. 1999	10.1111/j.1574-6968.1999.tb13647.x
Immune-associated traits measured in milk of Holstein-Friesian cows as proxies for blood serum measurements	Denholm SJ et al. 2018	10.3168/jds.2018-14825
Increase of lactoferrin concentration in mastitic goat milk	Chen PW et al. 2004	10.1292/jvms.66.345
Influence of subclinical mastitis and intramammary infection by coagulase-negative staphylococci on the cow milk peptidome	Addis MF et al. 2020	
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis	Sakemi Y et al. 2011	10.1017/S0022029910000828
Interrelationship between somatic cell count and acute phase proteins in serum and milk of dairy cows	Kováč G et al. 2007	10.2754/avb200776010051
Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with Staphylococcus aureus alpha-toxin	Riollet C et al. 2000	10.1007/s000110050621
Lactoferrin and IgG levels in ovine milk throughout lactation: Correlation with milk quality parameters	Navarro F et al. 2018	10.1016/j.smallrumres.2018.09.002
Lactoferrin and Immunoglobulin G Concentration in Bovine Milk from Cows with Subclinical Mastitis during the Late Lactation Period	Galfi A et al. 2016	
Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings	Galfi A et al. 2016	
Lactoferrin concentrations in bovine milk prior to dry-off	Newman KA et al. 2009	10.1017/S0022029909990033
Lactoferrin concentrations in goat milk throughout lactation	Hiss S et al. 2008	10.1016/j.smallrumres.2008.07.027
MicroRNA Milk Exosomes: From Cellular Regulator to Genomic Marker	Cintio M et al. 2020	10.3390/ani10071126
Mid-infrared prediction of lactoferrin content in bovine milk: potential indicator of mastitis	Soyeurt H et al. 2012	10.1017/S1751731112000791
Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation	Tedde V et al. 2019	10.1017/S0022029919000335
Milk haptoglobin detection based on enhanced chemiluminescence of gold nanoparticles	Nirala NR et al. 2019	10.1016/j.talanta.2019.01.027
Milk lactoferrin concentrations in anatolian buffaloes with and without subclinical mastitis	Ozenc E et al. 2019	
mRNA expression of immune factors and milk proteins in mammary tissue and milk cells and their concentration in milk during subclinical mastitis	Schmitz S et al. 2004	
Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving	Simoes PBA et al. 2018	10.1016/j.rvsc.2017.05.024
Plasma lactoferrin concentration measured by ELISA in healthy and diseased cows	Sato R et al. 2000	
Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation	Trevisi E et al. 2015	
Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification	Boehmer JL et al. 2010	10.3168/jds.2009-2526
Rapid biosensing of Staphylococcus aureus bacteria in milk	Peedel D et al. 2014	10.1039/c3ay42036a
Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis	Addis MF et al. 2017	10.3168/jds.2016-12110
Relationship of Late Lactation Milk Somatic Cell Count and Cathelicidin with Intramammary Infection in Small Ruminants	Puggioni GMG et al. 2020	10.3390/pathogens9010037
Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis	Kovacevic-Filipovic M et al. 2012	10.1016/j.vetimm.2011.10.015
Serum and milk concentrations of oxidant and anti-oxidant markers in dairy cows affected with bloody milk	Ismail ZB et al. 2020	
Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with Escherichia coli	Ma JL et al. 2011	10.1016/j.vetimm.2011.10.015
Serum haptoglobin-matrix metalloproteinase 9(Hp-MMP 9) complex as a biomarker of systemic inflammation in cattle	Bannikov GA et al. 2011	
Short communication: Production of antimicrobial peptide S100A8 in the goat mammary gland and effect of intramammary infusion of lipopolysaccharide on S100A8 concentration in milk	Purba FY et al. 2019	10.3168/jds.2018-15396
Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis	Jaeger S et al. 2017	10.1016/j.vetimm.2011.10.015
The Antisecretory Factor in Plasma and Breast Milk in Breastfeeding MothersA Prospective Cohort Study in Sweden	Gustafsson A et al. 2018	10.3390/nu10091227
The major acute phase proteins of bovine milk in a commercial dairy herd	Thomas FC et al. 2015	10.1186/s12917-015-0533-3
The proteomic advantage: Label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis	Boehmer JL et al. 2010	10.1016/j.vetimm.2010.10.004
The relationship between lactoferrin gene polymorphism and subclinical mastitis in awassi ewes	Alekish M et al. 2019	10.1016/j.vetimm.2012.06.017

Three novel single-nucleotide polymorphisms of complement component 4 gene(C4A) in Chinese Holstein cattle and their associations with milk performance traits and CH50	Yang Y et al. 2012	10.1016/j.vetimm.2011.11.010
Tumor necrosis factor-alpha and haptoglobin in the blood serum and mammary gland lymph from cows with acute clinical mastitis in comparison to healthy control animals	Hagen J et al. 2011	
Tumour necrosis factor-alpha(TNF-alpha) increases nuclear factor kappa B(NF kappa B) activity in and interleukin-8(IL-8) release from bovine mammary epithelial cells	Fitzgerald DC et al. 2007	10.1016/j.vetimm.2006.12.008
Ultrasensitive haptoglobin biomarker detection based on amplified chemiluminescence of magnetite nanoparticles	Nirala NR et al. 2020	10.1186/s12951-019-0569-9
Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes	Miglio A et al. 2013	10.1017/S0022029913000484
Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows	Gerardi G et al. 2009	10.1017/S0022029909990057

Table V. Scientific articles integrated by the expert reviewer

Title	Authors/Year	DOI
Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens	Pyörälä S et al. 2011	10.1136/vr.d1120. Epub 2011 May 9
Acute phase proteins in serum and milk from dairy cows with clinical mastitis	Eckersall PD et al. 2001	10.1136/vr.148.2.35
Acute phase proteins in the diagnosis of bovine subclinical mastitis	Shahabeddin S et al. 2009	10.1111/j.1939-165X.2009.00156.x
Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows.	Suojala L et al. 2008	10.1186/1751-0147-50-18
Changes in acute-phase proteins and cytokines in serum and milk whey from dairy cows with naturally occurring peracute mastitis caused by Klebsiella pneumoniae and the relationship to clinical outcome	Hisaeda K et al. 2011	10.1292/jvms.10-0403
Characterization of Haptoglobin Isotype in Milk of Mastitis-Affected Cows.	Upadhyaya I et al. 2016	10.3390/vetsci3040029
Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk quality	Åkerstedt M et al. 2009	10.1017/S0022029906002305
Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis.	Grönlund U et al. 2005	10.1051/vetres:2004063
Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples.	Åkerstedt M et al. 2007	10.1017/S0022029906002305
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis.	Sakemi Y et al. 2010	10.1017/S0022029910000828
Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle	Hussein HA et al. 2018	10.14202/vetworld.2018.34-41
Milk amyloid A: correlation with cellular indices of mammary inflammation in cows with normal and raised serum amyloid A	Mahony MCO et al. 2006	10.1016/j.rvsc.2005.05.005
Milk lactoferrin in heifers: influence of health status and stage of lactation.	Chaneton L et al. 2013	10.3168/jds.2012-6028
Natural variation in biomarkers indicating mastitis in healthy cows	Åkerstedt M et al. 2011	10.1017/S0022029910000786
Serum amyloid A as a marker of cow`s mastitis caused by Streptococcus sp.	Bochniarz M et al. 2020	10.1016/j.cimid.2020.101498
The value of the biomarkers cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis	Wollowski L et al. 2021	10.3168/jds.2020-18539. Epub 2020 Dec 23

Table VI. Inclusion and exclusion criteria used in the selection phases

1. Population: ruminant species
2. Matrix: milk
3. Object: Protein biomarkers
4. Measurement technique: Immunoassay
5. Language: English
6. Document types to be excluded: reviews, case reports, reports, book chapters, editorials, letters

Title	Title	Abstrac	Full
[C-reactive protein as a new parameter of mastitis]			
15-F-2t-Isoprostane Concentrations and Oxidant Status in Lactating Dairy Cattle with Acute Coliform Mastitis			
A genome-wide association study for natural antibodies measured in blood of Canadian Holstein cows			
A pilot study of acute phase proteins as indicators of bovine mastitis caused by different pathogens			
A proteomic perspective on the changes in milk proteins due to high somatic cell count			
A proteomics-based identification of putative biomarkers for disease in bovine milk			
Acute phase response in lame crossbred dairy cattle			
Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens			
Acute phase proteins in serum and milk from dairy cows with clinical mastitis			
Acute phase proteins in the diagnosis of bovine subclinical mastitis			
Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows.			
Advances in BHV1(IBR) research			
Assessment of milk quality trough microbiological an cytometric examination and determination of acute-phase proteins			
Binding of bovine lactoferrin to Streptococcus dysgalactiae subsp dysgalactiae isolated from cows with mastitis			
Biomarker based detection of subclinical mastitis by liquid phase blocking elisa			
Biosensor assay for determination of haptoglobin in bovine milk			
Bovine intra-mammary challenge with Streptococcus dysgalactiae spp. Dysgalactiae to explore the effect on the response of Complement activity			
Cathelicidin production and release by mammary epithelial cells during infectious mastitis			
Changes in acute-phase proteins and cytokines in serum and milk whey from dairy cows with naturally occurring peracute mastitis caused by Klebsiella pneumoniae and the relationship to clinical outcome			
Changes in the content of whey proteins during lactation in cow's milk with a different somatic cells count			
Characterization of Haptoglobin Isotype in Milk of Mastitis-Affected Cows.			
Comparative diagnosis of infectious bacteria in bovine milk			
Concentration of serum amyloid a and activity of ceruloplasmin in milk from cows with clinical and subclinical mastitis			
Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens			
Concentrations of acute-phase proteins in milk from cows with clinical mastitis caused by different pathogens			
Crosstalk between coagulation and inflammation in mastitis and metritis in dairy cows			
Cytokine and acute phase protein gene expression in repeated liver biopsies of dairy cows with a lipopolysaccharide-induced mastitis			
Detection of lactoferrin in bovine and goat milk by enzyme-linked immunosorbent assay			
Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis			
Development and validation of an ELISA for the quantification of bovine ITIH4 in serum and milk			
Development of an immunosensor assay for detection of haptoglobin in mastitic milk			
Dietary-induced negative energy balance has minimal effects on innate immunity during a Streptococcus uberis mastitis challenge in dairy cows during midlactation			
Dynamics of experimentally induced Staphylococcus epidermidis mastitis in East Friesian milk ewes			
Early pathogenesis and inflammatory response in experimental bovine mastitis due to Streptococcus uberis			
Early post parturient changes in milk acute phase proteins			
Effect of gestation length on the levels of five innate defence proteins in human milk			
Effect of intramammary infusion of tumour necrosis factor-alpha on milk protein composition and induction of acute-phase protein in the lactating cow			
Effects of induced energy deficiency on lactoferrin concentration in milk and the lactoferrin reaction of primary bovine mammary epithelial cells in vitro			
Effects of intrauterine infusion of bacterial lipopolysaccharides on the mammary gland inflammatory response in goats			
Effects of lactoferrin and milk on adherence of Streptococcus uberis to bovine mammary epithelial cells			
Elevated milk soluble CD14 in bovine mammary glands challenged with Escherichia coli lipopolysaccharide			

Escherichia coli and Staphylococcus aureus elicit differential innate immune responses following intramammary infection			
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture			
Evaluation of intramammary platelet concentrate efficacy as a subclinical mastitis treatment in dairy cows based on somatic cell count and milk amyloid A levels [Sütçü ıneklerde subklinik mastitis tedavisinde meme ıçi platelet konsantresi etkinliğinin somatik hücre sayımı ve süt amiloid a seviyeleri ıle değerlendirilmesi]			
Evaluation of milk cathelicidin for detection of bovine mastitis			
Evaluation of milk cathelicidin for detection of dairy sheep mastitis			
Expression of cathelicidins mRNA in the goat mammary gland and effect of the intramammary infusion of lipopolysaccharide on milk cathelicidin-2 concentration			
Expression of the peptidoglycan recognition protein, PGRP, in the lactating mammary gland			
Facile construction of a molecularly imprinted polymer-based electrochemical sensor for the detection of milk amyloid A			
Factors affecting the lactoferrin concentration in bovine milk			
Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis			
Generation of an anti-NAGase single chain antibody and its application in a biosensor-based assay for the detection of NAGase in milk			
Genetic variability of lactoferrin content estimated by mid-infrared spectrometry in bovine milk			
Gold nanoparticle size-dependent enhanced chemiluminescence for ultra-sensitive haptoglobin biomarker detection			
Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters			
Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk quality			
Haptoglobin and serum amyloid A in milk from dairy cows with chronic sub-clinical mastitis.			
Haptoglobin and serum amyloid A in relation to the somatic cell count in quarter, cow composite and bulk tank milk samples.			
Haptoglobin concentrations in blood and milk after endotoxin challenge and quantification of mammary Hp mRNA expression			
Identification of lactoferrin-binding proteins in bovine mastitis-causing Streptococcus uberis			
Immune-associated traits measured in milk of Holstein-Friesian cows as proxies for blood serum measurements			
Immunological responses of the lactating ovine udder following experimental challenge with Staphylococcus epidermidis			
Immunosensing system for rapid multiplex detection of mastitis-causing pathogens in milk			
Increase in milk metalloproteinase activity and vascular permeability in bovine endotoxin-induced and naturally occurring Escherichia coli mastitis			
Increase of lactoferrin concentration in mastitic goat milk			
Increased Epstein-Barr virus in breast milk occurs with subclinical mastitis and HIV shedding			
Influence of bacterial factors on proliferation of bovine mammary epithelial cells			
Influence of subclinical mastitis and intramammary infection by coagulase-negative staphylococci on the cow milk peptidome			
Innate immune response in experimentally induced bovine intramammary infection with Staphylococcus simulans and S. epidermidis			
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis			
Interrelationship between somatic cell count and acute phase proteins in serum and milk of dairy cows			
Kinetics of cells and cytokines during immune-mediated inflammation in the mammary gland of cows systemically immunized with Staphylococcus aureus alpha-toxin			
Kinetics of local and systemic isoforms of serum amyloid A in bovine mastitic milk			
Lactoferrin and IgG levels in ovine milk throughout lactation: Correlation with milk quality parameters			
Lactoferrin and Immunoglobulin G Concentration in Bovine Milk from Cows with Subclinical Mastitis during the Late Lactation Period			
Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings			
Lactoferrin concentrations in bovine milk prior to dry-off			
Lactoferrin concentrations in goat milk throughout lactation			
Low-level laser therapy attenuates LPS-induced rats mastitis by inhibiting polymorphonuclear neutrophil adhesion			
Mastitis detection: current trends and future perspectives			
Mastitis is associated with IL-6 levels and milk fat globule size in breast milk			
MicroRNA Milk Exosomes: From Cellular Regulator to Genomic Marker			
Mid-infrared prediction of lactoferrin content in bovine milk: potential indicator of mastitis			
Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle			
Milk amyloid A: correlation with cellular indices of mammary inflammation in cows with normal and raised serum amyloid A			
Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation			
Milk cytokines and subclinical breast inflammation in Tanzanian women: effects of dietary red palm oil or sunflower oil supplementation			
Milk haptoglobin detection based on enhanced chemiluminescence of gold nanoparticles			
Milk lactoferrin concentrations in anatolian buffaloes with and without subclinical mastitis			
Milk lactoferrin in heifers: influence of health status and stage of lactation.			
Milk prostaglandins and electrical conductivity in bovine mastitis			
mRNA expression of immune factors and milk proteins in mammary tissue and milk cells and their concentration in milk during subclinical mastitis			
Natural variation in biomarkers indicating mastitis in healthy cows			
Omic approaches to a better understanding of mastitis in dairy cows(Book Chapter)			
Pilot study into milk haptoglobin as an indicator of udder health in heifers after calving			
Plasma lactoferrin concentration measured by ELISA in healthy and diseased cows			
Pro-inflammatory cytokine profile in dairy cows: consequences for new lactation			
Proteomic analysis of the temporal expression of bovine milk proteins during coliform mastitis and label-free relative quantification			
Proteomics and pathway analyses of the milk fat globule in sheep naturally infected by Mycoplasma agalactiae provide indications of the in vivo response of the mammary epithelium to bacterial infection			
Purification of prostaglandin D synthase by ceramic- and size exclusion chromatography			
Rapid biosensing of Staphylococcus aureus bacteria in milk			

Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis			
Relationship between milk lactoferrin and etiological agent in the mastitic bovine mammary gland			
Relationship of Late Lactation Milk Somatic Cell Count and Cathelicidin with Intramammary Infection in Small Ruminants			
Serum amyloid A as an marker of cow`s mastitis caused by Streptococcus sp.			
Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis			
Serum and milk concentrations of oxidant and anti-oxidant markers in dairy cows affected with bloody milk			
Serum concentration and mRNA expression in milk somatic cells of toll-like receptor 2, toll-like receptor 4, and cytokines in dairy cows following intramammary inoculation with Escherichia coli			
Serum C-reactive protein in dairy herds			
Serum haptoglobin-matrix metalloproteinase 9(Hp-MMP 9) complex as a biomarker of systemic inflammation in cattle			
Short communication: Production of antimicrobial peptide S100A8 in the goat mammary gland and effect of intramammary infusion of lipopolysaccharide on S100A8 concentration in milk			
Staphylococcus aureus lipoteichoic acid triggers inflammation in the lactating bovine mammary gland			
Susceptibility of sows to experimentally induced Escherichia coli mastitis			
Technological interventions and advances in the diagnosis of intramammary infections in animals with emphasis on bovine population—a review			
Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis			
The acute-phase protein serum amyloid A3 is expressed in the bovine mammary gland and plays a role in host defence			
The antisecretory factor in plasma and breast milk in breastfeeding mothers—a prospective cohort study in Sweden			
The diagnostic value of determination of positive and negative acute phase proteins in milk from dairy cows with subclinical mastitis			
The Effect of Lipopolysaccharide-Induced Experimental Bovine Mastitis on Clinical Parameters, Inflammatory Markers, and the Metabolome: A Kinetic Approach			
The major acute phase proteins of bovine milk in a commercial dairy herd			
The production and characterization of anti-bovine CD14 monoclonal antibodies			
The proteomic advantage: label-free quantification of proteins expressed in bovine milk during experimentally induced coliform mastitis			
The relationship between lactoferrin gene polymorphism and subclinical mastitis in awassi ewes			
The relationship between the variants of the bovine MBL2 gene and milk production traits, mastitis, serum MBL-C levels and complement activity			
The value of the biomarkers cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis			
Three novel single-nucleotide polymorphisms of complement component 4 gene(C4A) in Chinese Holstein cattle and their associations with milk performance traits and CH50			
Tumor necrosis factor-alpha and haptoglobin in the blood serum and mammary gland lymph from cows with acute clinical mastitis in comparison to healthy control animals			
Tumour necrosis factor-alpha(TNF-alpha) increases nuclear factor kappa B(NF kappa B) activity in and interleukin-8(IL-8) release from bovine mammary epithelial cells			
Ultrasensitive haptoglobin biomarker detection based on amplified chemiluminescence of magnetite nanoparticles			
Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes			
Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows			

Table VII. Scientific papers not included in the qualitative analysis

Author	Species	Reason for exclusion
Åkerstedt et al. 2006	Cow	Single animal data
Åkerstedt et al. 2007	Cow	Missing data
Åkerstedt et al. 2011	Cow	Single animal data
Chaneton et al. 2008	Cow	Missing data
Chaneton et al. 2013	Cow	Missing data
Chen and Mao, 2004	Goat	Type of samples (bulk)
Eckersall et al. 2001	Cow	Unsuitable data *
Evkuran Dal et al. 2019	Cow	Missing data
Grönlund et al. 2005	Cow	Unsuitable data *-ATP for CMT?
Hiss et al. 2004	Cow	Missing data
Hiss et al. 2008	Goat	Aim of the study did not match the review question
Navarro et al. 2018	Sheep	Missing data
Newman et al. 2009	Cow	Missing data
Nirala and Shtenberg, 2019	Cow	Single animal data
O'Mahony et al. 2006	Cow	Missing data
Pedersen et al. 2003	Cow	Aim of the study did not match the review question
Simões et al. 2018	Cow	Aim of the study did not match the review question
Szczubiał et al. 2008	Cow	No full text available
Tan et al. 2012	Cow	Single animal data
Thomas et al. 2016	Cow	Aim of the study did not match the review question
Thomas et al. 2018	Cow	Samples identified as "all" (healthy status not specified)
Welbeck et al. 2001	Human	No dairy ruminant species
Zhang et al. 2014	Goat	Missing data

Table VIII. The 33 articles selected for the qualitative synthesis

Title	Authors/Year	DOI
Acute phase proteins in milk in naturally acquired bovine mastitis caused by different pathogens	Pyörälä S et al. 2011	10.1136/vr.d1120. Epub 2011 May 9
Acute phase proteins in the diagnosis of bovine subclinical mastitis	Shahabeddin S et al. 2009	10.1111/j.1939-165X.2009.00156.x
Acute phase response in two consecutive experimentally induced E. coli intramammary infections in dairy cows.	Suojala L et al. 2008	10.1186/1751-0147-50-18
Changes in the content of whey proteins during lactation in cow's milk with a different somatic cells count	Sobczuk-Szul M et al. 2014	
Concentration of serum amyloid A and ceruloplasmin activity in milk from cows with subclinical mastitis caused by different pathogens	Szczubiał M et al. 2012	10.2478/v10181-011-0149-x
Concentrations of acute-phase proteins in milk from cows with clinical mastitis caused by different pathogens	Dalanezi FM et al. 2020	10.3390/pathogens9090706
Detection of lactoferrin in bovine and goat milk by enzyme-linked immunosorbent assay	Chen PW et al. 2004	10.38212/2224-6614.2653
Determination of milk and blood concentrations of lipopolysaccharide-binding protein in cows with naturally acquired subclinical and clinical mastitis	Zeng R et al. 2009	10.3168/jds.2008-1636
Evaluation of a bovine cathelicidin ELISA for detecting mastitis in the dairy buffalo: Comparison with milk somatic cell count and bacteriological culture	Puggioni GMG et al. 2020	10.1016/j.rvsc.2019.11.009
Evaluation of milk cathelicidin for detection of bovine mastitis	Addis MF et al. 2016	10.3168/jds.2016-11407
Evaluation of milk cathelicidin for detection of dairy sheep mastitis	Addis MF et al. 2016	10.3168/jds.2015-10293
Factors affecting the lactoferrin concentration in bovine milk	Cheng JB et al. 2008	10.3168/jds.2007-0689
Factors associated with concentrations of select cytokine and acute phase proteins in dairy cows with naturally occurring clinical mastitis	Wenz JR et al. 2010	10.3168/jds.2009-2819
Haptoglobin and lactate dehydrogenase measurements in milk for the identification of subclinically diseased udder quarters	Hiss S et al. 2007	10.17221/1879-VETMED
Haptoglobin and serum amyloid A in bulk tank milk in relation to raw milk quality	Åkerstedt M et al. 2009	10.1017/S0022029906002305
Immunological responses of the lactating ovine udder following experimental challenge with Staphylococcus epidermidis	Winter P et al. 2002	
Increase of lactoferrin concentration in mastitic goat milk	Chen PW et al. 2004	10.1292/jvms.66.345
Interleukin-6 in quarter milk as a further prediction marker for bovine subclinical mastitis	Sakemi Yet al. 2011	10.1017/S0022029910000828
Interrelationship between somatic cell count and acute phase proteins in serum and milk of dairy cows	Kováč G et al. 2007	10.2754/avb200776010051
Lactoferrin and Immunoglobulin G Concentration in Bovine Milk from Cows with Subclinical Mastitis during the Late Lactation Period	Galfi A et al. 2016	
Lactoferrin concentrations in bovine milk during involution of the mammary glands, with different bacteriological findings	Galfi A et al. 2016	
Milk amyloid A as a biomarker for diagnosis of subclinical mastitis in cattle	Hussein HA et al. 2018	10.14202/vetworld.2018.34-41
Milk cathelicidin and somatic cell counts in dairy goats along the course of lactation	Tedde V et al. 2019	10.1017/S0022029919000335
Milk lactoferrin concentrations in anatolian buffaloes with and without subclinical mastitis	Ozenc E et al. 2019	
Relationship between milk cathelicidin abundance and microbiologic culture in clinical mastitis	Addis MF et al. 2017	10.3168/jds.2016-12110
Relationship of Late Lactation Milk Somatic Cell Count and Cathelicidin with Intramammary Infection in Small Ruminants	Puggioni GMG et al. 2020	10.3390/pathogens9010037
Serum amyloid A as an marker of cow`s mastitis caused by Streptococcus sp.	Bochniarz M et al. 2020	10.1016/j.cimid.2020.101498
Serum amyloid A isoforms in serum and milk from cows with Staphylococcus aureus subclinical mastitis	Kovačević-Filipović M et al. 2003	10.1016/j.vetimm.2011.10.015
Test characteristics of milk amyloid A ELISA, somatic cell count, and bacteriological culture for detection of intramammary pathogens that cause subclinical mastitis	Jaeger S et al. 2017	10.3168/jds.2016-12446
The diagnostic value of determination of positive and negative acute phase proteins in milk from dairy cows with subclinical mastitis	Shirazi-Beheshtiha SH et al. 2012	
The major acute phase proteins of bovine milk in a commercial dairy herd	Thomas FC et al. 2015	10.1186/s12917-015-0533-3
The value of the biomarkers cathelicidin, milk amyloid A, and haptoglobin to diagnose and classify clinical and subclinical mastitis	Wollowski L et al. 2021	10.3168/jds.2020-18539. Epub 2020 Dec 23

Use of milk amyloid A in the diagnosis of subclinical mastitis in dairy ewes	Miglio A et al. 2013	10.1017/S0022029913000484
Use of serum amyloid A and milk amyloid A in the diagnosis of subclinical mastitis in dairy cows	Gerardi G et al. 2009	10.1017/S0022029909990057

Table IX Bias assessment of eligible records for qualitative analysis

Scientific article	Animal Selection	Index Test	Reference Standard	Flow and Timing
Addis et al. 2016a	Low	Unclear	Unclear	Low
Addis et al. 2016b	Low	Low	Unclear	Low
Addis et al. 2017	High	High	Low	Low
Åkerstedt et al. 2009	Low	High	High	Low
Bochniarz et al. 2020	Low	High	Low	Unclear
Chen et al. 2004	Low	High	High	Low
Cheng et al. 2008	High	High	Low	High
Dalanezi et al. 2020	Low	High	High	Low
Galfi et al. 2016a	High	High	High	Low
Galfi et al. 2016b	Low	High	High	Low
Gerardi et al. 2009	High	High	Low	High
Hiss et al. 2007	High	High	Low	Low
Hussein et al. 2018	High	High	Low	High
Jaeger et al. 2017	Low	High	Low	High
Kováč et al. 2007	High	High	Low	High
Kovačević-Filipović et al. 2012	Low	High	Low	Low
Miglio et al. 2013	Low	High	Low	High
Özenç et al. 2019	High	High	Low	High
Puggioni et al. 2020a	Low	Unclear	Low	Low
Puggioni et al. 2020b	Low	Unclear	Low	Low
Pyorala et al. 2011	Low	High	High	Low
Safi et al. 2009	High	High	Low	High
Sakemi et al. 2011	Low	High	Low	High
Shirazi-Beheshtiha et al. 2011	Low	High	Low	Low
Sobczuk-Szul et al. 2014	High	High	High	Low
Suojala et al. 2008	High	High	Low	Low
Szczubiał et al. 2012	High	High	Low	Low
Tedde et al. 2019	Low	High	Low	Low
Thomas et al. 2015	Low	High	High	Low
Wenz et al. 2010	High	High	Low	High
Winter and Colditz 2002	High	High	High	Low
Wollowski et al. 2021	High	High	Low	High
Zeng et al. 2009	Low	High	Low	Low

Table X Applicability of eligible records for qualitative analysis

Scientific article	Animal Selection	Index Test	Reference Standard
Addis et al. 2016a	Low	Low	Low
Addis et al. 2016b	Low	Low	Low
Addis et al. 2017	Low	Low	Low
Åkerstedt et al. 2009	Low	Low	Low
Bochniarz et al. 2020	Low	Low	Low
Chen et al. 2004	Low	Low	Low
Cheng et al. 2008	High	High	Low
Dalanezi et al. 2020	Low	High	Low
Galfi et al. 2016a	High	High	Low
Galfi et al. 2016b	Low	High	High
Gerardi et al. 2009	Low	Low	Low
Hiss et al. 2007	Low	Low	Low
Hussein et al. 2018	Low	Low	Low
Jaeger et al. 2017	Low	Low	Low
Kováč et al. 2007	Low	Low	Low
Kovačević-Filipović et al. 2012	Low	Low	Low
Miglio et al. 2013	Low	Low	Low
Özenç et al. 2019	Low	Low	Low
Puggioni et al. 2020a	Low	Low	Low
Puggioni et al. 2020b	Low	Low	Low
Pyörälä et al. 2011	Low	Low	High
Safi et al. 2009	Low	Low	Low
Sakemi et al. 2011	Low	Low	Low
Shirazi-Beheshtiha et al. 2011	Low	Low	Low
Sobczuk-Szul et al. 2014	High	Unclear	Low
Suojala et al. 2008	Low	Unclear	Low
Szczubiał et al. 2012	Low	Low	Low
Tedde et al. 2019	Low	High	Low
Thomas et al. 2015	Low	Low	Low
Wenz et al. 2010	Low	Low	Low
Winter and Colditz 2002	High	Low	Low
Wollowski et al. 2021	Low	Low	Low
Zeng et al. 2009	Low	Unclear	Low

Part III: Feeding Pre-weaned calves with waste milk: what are the consequences on gut health, microbiome development, and antimicrobial resistance?

Waste milk (WM) is a major by-product of the dairy industry that cannot be marketed for human consumption. The discarded WM may serve as a good feed source for dairy calves because of its high nutrient content. Using WM for feeding calves might expose newborn calves to infectious agents and residual antimicrobials that WM can contain and may negatively influence the animals' gut microbiota. We assessed the impact of WM on the calf intestinal microbiome by analyzing the effect on intestinal health and fecal microbiota of calves fed with WM in the first weeks of life in an 8-week trial. The study was performed on a commercial dairy farm in Northern Italy with a long-standing collaboration with the University of Milan. The trial enrolled twelve consecutive born male calves at birth between March 11 and April 22, 2019. Following the three-day colostrum administration, six calves were allocated to the BM group and six to the WM group according to birth order. For two weeks (Wk0–Wk2), BM calves were fed twice a day with 2 L of fresh unpasteurized BM, while WM calves were fed twice a day with 2 L of an unpasteurized WM lot that was prepared, standardized, and characterized before the beginning of the trial. These first two weeks were followed by six weeks of feeding commercial milk replacer and pelleted starter feed. During the whole trial, calves were sampled at six experimental time points (Wk0–Wk8): at birth and on the third day (Wk0), 10th day (Wk1), 17th day (Wk2), 31st day (Wk4), 45th day (Wk6), and 59th day of life (Wk8). At each time point, 12 fecal samples were collected, refrigerated, brought to the laboratory within 12 h, and stored at −20°C to assess the possible selection and maintenance of resistant bacteria in the gut. The WM preparation and administration procedures, as well as the composition of WM, BM, and milk replacer, are detailed in sections Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk. The body weight of WM calves was significantly lower than that of BM calves, and the majority of WM calves had diarrhea episodes in the first two weeks of the trial (5/6 WM and 1/6 BM). Based on 16S rRNA gene analysis, WM calves had a lower fecal microbiota alpha diversity than that BM calves at Wk4 ($p < 0.02$), and the fecal microbiota beta diversity of the two calf groups was also significantly different at Wk4 ($p < 0.05$). In the fecal microbiota taxonomy of WM and BM calves, significant differences were present in relative normalized operational taxonomic unit (OTU) levels, affecting five phyla, seven classes, eight orders, 19 families, and 47 genera (Figure 17). Our results suggest that adding to the risk of increasing antibiotic resistance, feeding pre-weaned calves with WM is related to a higher incidence of calf diarrhea and relevant changes in the fecal microbiota composition.

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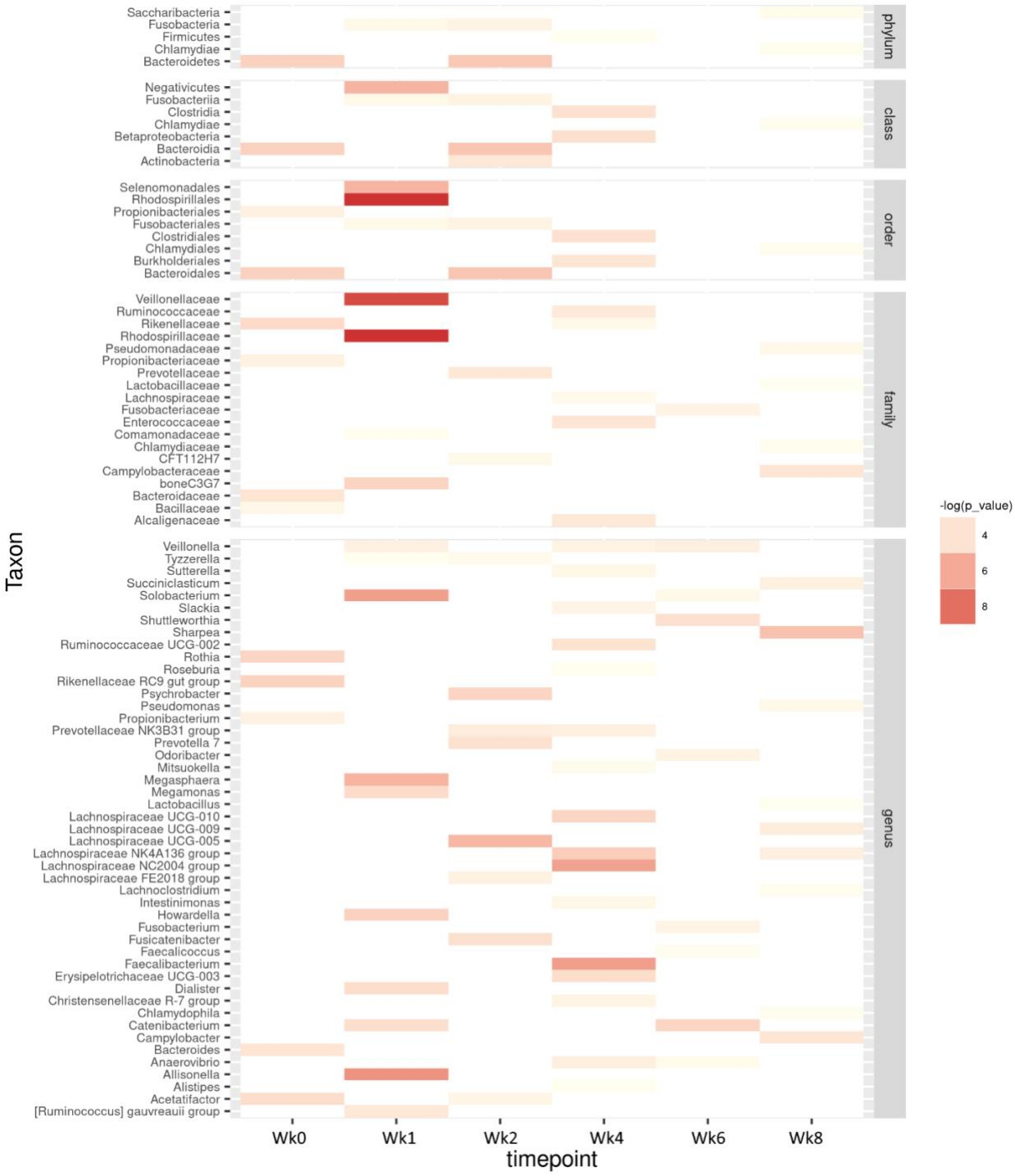

Figure 17: Statistical significance of the differences in normalized OTU abundances between WM and BM calves reported as a heatmap. The intensity of the red color increases with statistical significance (Reproduced from Penati et al., 2021)

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Feeding pre-weaned calves with waste milk containing antibiotic residues is related to a higher incidence of diarrhea and alterations in the fecal microbiota

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Abstract

Cows receiving antibiotics for intra-mammary infection (IMI) produce milk that cannot be marketed. This is considered waste milk (WM), and a convenient option for farmers is using it as calf food. Adding to the risk of selecting resistant bacteria, residual antibiotics might interfere with gut microbiome development and influence gastrointestinal health. We assessed the longitudinal effect of unpasteurized WM containing residual cefalexin on calf intestinal health and fecal microbiota in an 8-week trial. After three days of colostrum, six calves received WM, and six calves received bulk tank milk (BM) for two weeks. All 12 calves received milk substitute and starter feed for the following six weeks. Every week for the first two weeks and every two weeks for the remaining six weeks, we subjected all calves to clinical examination and collected rectal swabs to investigate the fecal microbiota composition. Most WM calves had diarrhea episodes in the first two weeks of the trial (5/6 WM and 1/6 BM), and their body weight was significantly lower than that of BM calves. Based on 16S rRNA gene analysis, WM calves had a lower fecal microbiota alpha diversity than BM calves, with the lowest *p*-value at Wk4 ($p < 0.02$) two weeks after exposure to WM. The fecal microbiota beta diversity of the two calf groups was also significantly different at Wk4 ($p < 0.05$). Numerous significant differences were present in the fecal microbiota taxonomy of WM and BM calves regarding relative normalized operational taxonomic unit (OTU) levels, affecting five phyla, seven classes, eight orders, 19 families, and 47 genera. At the end of the trial, when six weeks had passed since exposure to WM, the phyla Bacteroidetes, Firmicutes, and Saccharibacteria were lower,

while Chlamydiae were higher in WM calves. Notably, WM calves showed a decrease in beneficial taxa such as *Faecalibacterium*, with a concomitant increase in potential pathogens such as *Campylobacter*, *Pseudomonas*, and *Chlamydophila* spp. In conclusion, feeding pre-weaned calves with unpasteurized WM-containing antibiotics is related to a higher incidence of neonatal diarrhea. It leads to significant changes in the fecal microbiota composition, further discouraging this practice despite its short-term economic advantages.

Keywords: calf, microbiome, milk, antibiotic residues, gut microbiome, mastitis

Introduction

Waste milk (WM) includes low-quality colostrum, transition or post-colostral milk, milk from cows treated for mastitis and other diseases, milk with high somatic cell count (SCC), and other unsalable milk (Ricci et al., 2017). According to European food safety regulations (such as EC Regulation 853 of 2004), this milk is not allowed for direct human consumption or processing into dairy products, with no specific provisions for other uses. Given the clear economic and practical advantages, WM is widely used by farmers as calf food (Ricci et al., 2017; Brunton et al., 2012). Several countries are issuing guidelines discouraging this practice (i.e., European Commission notice 2015/C 299/04) (Ricci et al., 2017), as the potential presence of anti-microbial residues may increase the risk of maintaining and spreading antimicrobial resistance gene pools on the dairy farm and the environment (Deng et al., 2017; Thames et al., 2012) and expose newborn calves to intestinal diseases (Kogut et al. 2019; Kogut et al. 2016; Malmuthuge et al., 2017). A further potential issue is the interference of antibiotics and microbial pathogens with the gut microbiome's physiological development in growing calves, with possible consequences on their future health and production performances (Kogut et al., 2019; Kogut et al. 2016; Malmuthuge et al., 2017). When antibiotics are administered to adult individuals with a mature gut microbiome, microbial diversity has been shown to decrease significantly, but resilience mechanisms slowly restore the original condition once antibiotics are removed (Palleja et al., 2018). Exposure to antimicrobials at an early age may lead to permanent shifts in microbial composition and functions with consequent long-term metabolic alterations (Cho et al., 2012; Cox et al., 2014; Greenwood et al., 2014). Adding to the increased risk of selecting antimicrobial resistance traits, feeding calves with milk containing antimicrobials in the first weeks of life might compromise their intestinal microbiome development impacting gut immunity, gastrointestinal well-being, and ability to metabolize nutrients efficiently (Maynou et al., 2019; Pereira et al., 2016). Given its relevance for the dairy industry, previous studies have assessed the impact of WM on calf health and the gut microbiome (Deng et al., 2017, Maynou et al., 2019; Pereira

et al., 2016), investigating subtherapeutic levels of antibiotics spiked into milk (Pereira et al., 2016) or milk replacer (Maynou et al., 2019; Yousif et al., 2018) and pasteurized WM with antibiotic residues at unknown concentrations (Deng et al., 2017, Edrington et al., 2012, Zou et al., 2017). These studies demonstrated that short-term changes in the microbial taxonomy occur following WM ingestion, but these are generally limited to disruptions that do not go beyond the genus level (Pereira et al., 2016). These studies investigated low or undetermined antibiotic residues and assessed only the time frame of WM feeding. With these premises, we evaluated the impact of WM obtained from cows receiving intra-mammary cefalexin on calf intestinal health and fecal microbiota diversity and taxonomy during two weeks of feeding and up to six weeks after the removal of WM from the diet to reduce variability, colostrum, and WM were standardized and characterized before feeding them to calves. The two-step, 8-week trial included 12 dairy calves enrolled in a commercial farm and managed with standard procedures. For the first two weeks, six calves received WM, and six received bulk tank milk (BM); for the following six weeks, all calves received the same weaning diet with milk whey and starter feed. We conducted a complete clinical evaluation every week for the first two weeks and biweekly for the next six weeks and collected fecal swabs to investigate the fecal microbiota composition.

Materials and methods

Farm Description and Ethics Statement

The study was performed on a commercial dairy farm in Northern Italy with a long-standing collaboration with the University of Milan. The farm included 390 lactating Italian Friesian cows. The herd was accredited free from infectious bovine rhinotracheitis (IBR), vaccinated for neonatal diarrhea agents [Rotavec Corona®, MSD Animal Health S.r.l., Segrate (MI), Italy], and type-1 and type-2 bovine viral diarrhea virus (BVDV) (Bovela®, Boehringer Ingelheim, Milan, Italy). The farm was followed by our University Hospital Clinic and was selected for its very low prevalence of neonatal calf diarrhea (NCD) in the previous three months (<1% of cases between newborn calves). The research protocols were reviewed and approved by the Institutional Committee for Animal Care of the University of Milan (protocol number 78_2018). The trial was carried out between March 2019 and June 2019.

Design of the Feeding Trial and Sample Collection

The trial structure is illustrated in Figure 1. Twelve consecutive born male calves were enrolled at birth between March 11 and April 22, 2019. The calves were separated from the dam immediately after birth and received 3 L of the same standardized first colostrum within 6–8 h, followed by 2 L after 8–12 h. During the second and third days of life, calves were fed two times daily with 2.5L of the same standardized second-day and third-day transition milk (TM), respectively. Colostrum and TM, preparation and administration procedures, are detailed in section Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk. Starting from the fourth day of life, six calves were allocated to the BM group and six to the WM group according to birth order. For two weeks (Wk0–Wk2; Figure 1), BM calves were fed twice a day with 2 L of fresh unpasteurized BM, while WM calves were fed twice a day with 2 L of an unpasteurized WM lot that was prepared, standardized, and characterized before the beginning of the trial. For the following six weeks (Wk2–Wk8; Figure 1), all calves were fed twice a day with 6 L of a commercial milk replacer (Emme Erre Flash 22,5, Tredi Italia S.r.l., Cremona, Italy), and pelleted starter feed (Fly Start, Cortal Extrasoy S.p.A., Cittadella, PD, Italy) was available *ad libitum*. In the first two weeks, the calves were housed in individual hutches, while in the last six weeks, they were kept in two separate collective pens, one for each experimental group. The WM preparation and administration procedures, as well as the composition of WM, BM, and milk replacer, are detailed in sections Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk. At birth and on the third day (Wk0), 10th day (Wk1), 17th day (Wk2), 31st day (Wk4), 45th day (Wk6), and 59th day of life (Wk8), all calves were submitted to a complete clinical examination (Pravettoni et al., 2017) as detailed in section Clinical Examination and Calf Growth Measurements. At each time point, duplicate rectal swabs were collected, refrigerated, brought to the laboratory within 12 h, and stored at −20◦C until DNA extraction.

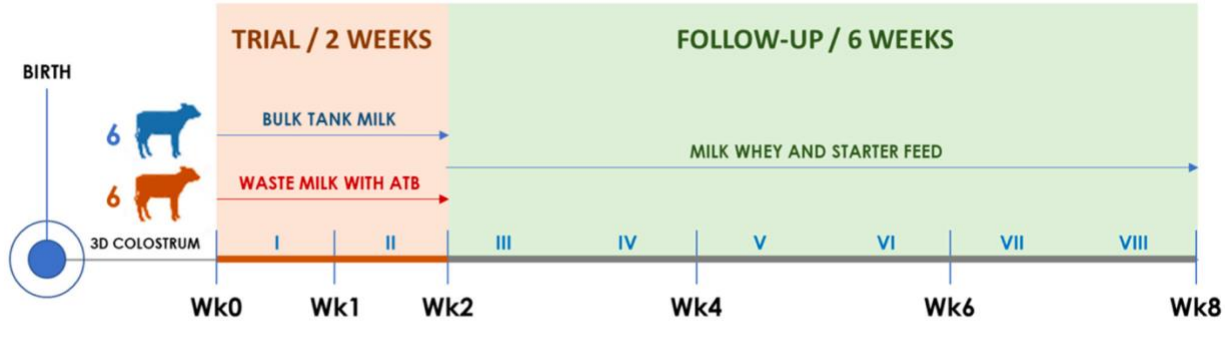

FIGURE 1 | Timeline of the trial illustrating calf groups, diets, and sampling times. Roman numerals indicate weeks. The timing of clinical visits and rectal swab collection is shown according to the trial week, as follows. Wk0: third day of life; Wk1: 10th day of life; Wk2: 17th day of life; Wk4: 31st day of life; Wk6: 45th day of life; Wk8: 59th day of life.

Colostrum, Transition Milk, Waste Milk, and Bulk Tank Milk

To eliminate possible variables related to colostrum or TM, a pooling strategy was applied as follows. Six liters of good-quality colostrum (Brix >22%) were milked from each of the ten cows and stored in 500 ml bottles, 12 for each cow. The bottles were identified as colostrum, labeled with the cow number, and frozen at −20°C. Then, 6 L of the second and third milking of the same cows (TM) were collected in 500 ml bottles, 12 for each cow. The bottles were identified as second-day or third-day TM, labeled with the cow number, and frozen at −20°C. For colostrum administration, the 3-L morning feeding of each calf was prepared by defrosting and pooling six 500-ml aliquots belonging to cows 1–6, while the 2-L afternoon feeding was prepared by defrosting and pooling four 500-ml aliquots belonging to cows 7–10. The aliquots were gently thawed in a water bath at 45°C for 30 min, mixed, and administered at 35– 40°C by oroesophageal tubing. The second-day TM and third-day TM were prepared by mixing aliquots 1–5 for the morning dose (2.5L for each calf) and aliquots 6–10 for the afternoon dose (2.5L for each calf) of the respective TM. In this way, all calves received the same colostrum and TM before the start of the feeding trial. WM was obtained from five cows affected by chronic mastitis (A–E), selected based on a previous bacteriological culture result according to the National Mastitis Council (NMC) guidelines (Middleton et al., 2017). Ten microliters of milk were spread on blood agar plates (5% defibrinated sheep blood), incubated at 37◦C, and examined after 24 and 48 h. Colonies were identified based on size, Gram stain, morphology, and hemolysis pattern. The SCC was determined using an automated counter (Bentley Somacount 150, Bentley Instruments, Chaska, MN, USA). The milk collected from the five cows had the following characteristics in terms of SCC and isolated bacteria: cow A, SCC 312,000 cells/ml, *Bacillus* spp.; cow B, SCC 901,000 cells/ml, non-*aureus* staphylococci (NAS); cow C, SCC 239,000 cells/ml, *Staphylococcus aureus*; cow D, SCC 5,045,000 cells/ml, *Bacillus* spp.; cow E, SCC 454,000 cells/ml, NAS. The five cows were subjected to the intramammary administration of 210 mg cefalexin monohydrate (Rilexine 200 T lactation, Virbac S.r.l.) in each quarter for four consecutive milkings, and the milk was collected at each following milking time for a total of 336L. All the milk was maintained in a refrigerated tank for 36 h from the first to the fourth milking, mixed, aliquoted in 2-L aluminum bags (Perfect Udder® bags, Dairy Tech Inc.), and stored at −20°C until needed. This collection, mixing, and aliquoting procedure ensured the generation of a uniform pooled WM. WM bags were gently thawed in a water bath at no more than 45°C for 45min and fed to calves at a temperature ranging from 35 to 40°C. BM was collected fresh from the commercial milk tank. WM and BM were subjected to the determination of total fat, protein, and lactose according to the ISO 9622:2013 (IDF 141) methods and tested for the presence of inhibitors by the Delvotest® SP NT (DSM). WM was further evaluated in triplicate by liquid chromatography–high-resolution mass

spectrometry (LC-HRMS) for antibiotic residue detection and quantitation as described by Chiesa et al. (Chiesa et al., 2020). The commercial milk replacer contained milk whey, whey proteins, vegetable oils (coconut, palm), hydrolyzed wheat protein, pregelatinized wheat flour, dextrose, butyric acid esters added with vitamins, oligo-elements, and stabilizers of the intestinal flora *Enterococcus faecium* DSM 7134 and *Lactobacillus rhamnosus* DSM 7133 at 1 × 109 CFU/kg. The powder was reconstituted according to the manufacturer's instructions (125 g/L of powder).

Clinical Examination and Calf Growth Measurements

Clinical examination and calf growth measurements were performed at the six experimental time points (Wk0–Wk8; Figure 1) by an expert bovine practitioner (GS). At 24 h from birth and on the third day of life, each calf's serum total protein concentration (STP) was measured to assess the correct transfer of passive immunity (Tyler et al., 1996). A blood sample was collected in a 9-ml tube without anticoagulant from the jugular vein. Samples were allowed to clot, centrifuged at 20°C for 10 min at 900 g, and the STP was measured with a handle refractometer. The calf growth rate was estimated using a heart-girth measuring tape pulled snuggly around the thorax, just caudal to the forelimbs. Obtained measurements were then used to estimate body weight (BW) following the equation proposed by Heinrichs et al. (Heinrichs et al., 1992). Diarrhea was defined as when a calf had visibly watery feces (fecal consistency that permitted feces to run through slightly opened fingers). When a diarrhea episode was detected, fecal samples were collected and submitted to routine diagnostic tests at the local animal health institution (Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia-Romagna) for the primary agents of NCD: rotavirus and coronavirus by real-time PCR and bacteriological agents by culture.

DNA Extraction and Generation of 16S rDNA Data

Rectal swabs were thawed, and DNA was extracted using a QIAmp DNA Stool kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions with a minor modification. The rectal swabs were dissolved in 1 ml Buffer ASL and shaken at 1,000 rpm (Mixing Block MB-102, CaRlibiotech S.r.l. Rome, Italy) continuously until the stool samples were homogenized. DNA quality and quantity were assessed with a NanoDrop ND- 1000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA), and the isolated DNA was stored at −20°C until use. Bacterial DNA was amplified by targeting the V3–V4 hypervariable regions of the 16S rRNA gene (Caporaso et al., 2011). PCR amplification of each sample was performed in a 25-µl volume. A total of 12.5 µl of KAPA HIFI Master Mix 2× (Kapa Biosystems, Inc., MA, USA) was used. Then, 0.2 µl of each primer (100 µM) was added to 2 µl of genomic DNA (5 ng/µl). Blank controls (no DNA template) were also

included. Amplification and library quantification were carried out as described previously (Biscarini et al., 2020).

Bioinformatic Processing

Demultiplexed paired-end reads from 16S rRNA-gene sequencing were first checked for quality using FastQC (Andrews, 2010) for an initial assessment. Forward and reverse paired-end reads were joined into single reads using the C++ program SeqPrep (John, 2011). After joining, reads were filtered for quality based on (i) the maximum three consecutive low-quality base calls (Phred <19) allowed; (ii) the fraction of consecutive high-quality base calls (Phred >19) in a read over total read length ≥0.75; (iii) no "N"-labeled bases (missing/uncalled) allowed. Reads that did not match all the above criteria were filtered out. All remaining reads were combined in a single FASTA file to identify and quantify operational taxonomic units (OTUs). Reads were aligned against the SILVA closed reference sequence collection release 123, with 97% cluster identity (Quast et al., 2013; Yilmaz et al., 2014) applying the CD-HIT clustering algorithm (Li et al., 2006). A predefined taxonomy map of reference sequences to taxonomies was then used for taxonomic identification along the main taxa ranks down to the genus level (domain, phylum, class, order, family, and genus). By counting the abundance of each OTU, the OTU table was created and then grouped at each phylogenetic level. OTUs with total counts lower than 10 in fewer than two samples were filtered out. All the above steps, except the FastQC reads quality check, were performed with the Quantitative Insights into Microbial Ecology (QIIME) open-source bioinformatics pipeline for microbiome analysis (Caporaso et al., 2018). More details on the command lines used to process 16S rRNA-gene sequence data can be found in Biscarini et al. (Biscarini et al., 2018). The 16S rRNA-gene sequencing reads were processed using the QIIME pipeline (Caporaso et al., 2018) to estimate the most diversity indices. The Abundance-based Coverage Estimator (ACE) index and sample-based rarefaction were estimated using Python (https://github. com/filippob/Rare-OTUs-ACE.git) and R (https://github.com/ filippob/sampleBasedRarefaction) scripts. Plots were generated using the ggplot2 R package (Wickham et al., 2009). Additional data handling and statistical analysis were performed using the R environment for statistical computing (R Core Team, 2018) and Microsoft Excel.

Alpha and Beta Diversity Indices

The fecal microbiota diversity was assessed within (alpha diversity) and across (beta diversity) samples. All indices (alpha and beta diversity) were estimated from the complete OTU table (at the OTU level) and filtered for OTUs with more than ten total counts distributed in at least two samples. Besides the number of observed OTUs directly counted from the OTU table, within-sample microbial richness, diversity, and evenness were estimated using the following indices: Chao1 and ACE for richness; Shannon, Simpson, and Fisher alpha for diversity (Chao, 1984; Chao, 1992; Fisher et al., 1943; Shannon, 1948; Simpson, 1949); Simpson E and Pielou J (Shannon evenness) for evenness (Smith et al., 1996). The across-sample microbiota diversity was quantified by calculating Bray–Curtis dissimilarities (Bray et al., 1957). Before calculating the Bray–Curtis dissimilarities, OTU counts were normalized for uneven sequencing depth by cumulative sum scaling CSS (Paulson et al., 2013). Among-groups (BM vs. WM) and pairwise Bray–Curtis dissimilarities were evaluated non-parametrically using the permutational analysis of variance (999 permutations) (Anderson et al., 2001). Details on the calculation of the mentioned alpha and beta diversity indices can be found in Supplementary File 1 and Biscarini et al. (Biscarini et al., 2018).

Statistical Analysis

The differences between feeding groups were evaluated with SPSS 25.0 (IBM). The distribution of continuous variables was analyzed with the Shapiro–Wilk test. Since the distribution was not normal, data were compared with a non-parametric Mann–Whitney *U*-test. Categoric variables were compared with contingency tables and the Fisher's exact test (2 × 2 tables), calculating the odds ratio. Statistical significance was considered for $p < 0.05$. For the microbiome analysis, differences between groups (WM, BM) along time points in terms of OTU abundances and alpha diversity indices were evaluated with a linear model of the following form:

$$y\_ij = mu + treatment\_j + e\_ij \quad (1)$$

where y_ij is the abundance (counts) or index value for each taxonomy (OTU) and alpha diversity metric in animal I belonging to treatment group j, treatment_j is either WM or BM, and e_ij are the residuals of the model. From model (1), *p*-values were obtained to identify those OTUs and alpha diversity indices that significantly differed between treatments along the six-time points of the experiment/trial. Alpha diversity indices: value = mu + group + e, within time point.

Results

Composition of Waste Milk and Bulk Tank Milk

WM had the following gross composition: SCC 450,000 cells/ml; fat 3.7%; protein 3.6%; lactose 4.7%; microbial inhibitors: present. According to HPLC-MS/MS (Chiesa et al., 2020), WM had a residual cefalexin concentration of 727 ppb (727 ng/ml). The mean ± SD composition of BM, based on the routine 10-day measurements received by the farm during its use in the trial, was the following: SCC 284,000 ± 38,742.74 cells/ml; fat 4.23% ± 0.06; protein 3.60% ± 0.00; lactose 4.97 ± 0.06; microbial inhibitors: absent.

Clinical Findings

During the first two weeks of the trial, five out of six (83.33%) WM calves and one out of six (16.67%) BM calves had at least one diarrhea episode. Diarrhea occurred without general impairment of clinical conditions (calves stood securely, presented a strong suckle reflex, and dehydration was <3–5%) (Boccardo et al., 2017). Diarrheic calves were treated with oral rehydration solution (ORS) containing 4 g sodium chloride, 20 g dextrose, 3 g potassium bicarbonate, and 3 g sodium propionate between milk feedings, as described by Boccardo et al. (Boccardo et al., 2017). According to Constable guidelines (Constable, 2004), antibiotic treatment was omitted because clinical conditions were not severe, no bacterial pathogens of NCD were detected by fecal analysis, and all calves presented an adequate transfer of passive immunity [BM group: 60 g/L of STP, 25% interquartile range (IQR) 58.5 g/L, 75% IQR 61.5 g/L; WM group: 64 g/L of STP, 25% IQR 57.5 g/L, 75% IQR 69 g/L]. During the study period, there were no mortality cases. At Wk0, the calves enrolled in the BM and WM groups had estimated median weights of 45.41 (25% IQR 43.27; 75% IQR 47.32) and 41.94 (25% IQR 40.61; 75% IQR 48.04), respectively. The difference in weight between the two calf groups at the beginning of the trial was not statistically significant ($p = 0.29$). At Wk1, the difference in estimated weight was significant ($p < 0.05$) and remained so until the end of the trial (Wk8), when the BM and WM groups had estimated median weights of 85.24 (25% IQR 78.50; 75% IQR 86.50) and 69.99 (25% IQR 62.69; 75% IQR 76.81), respectively.

Impact of Waste Milk on Fecal Microbiota Diversity

Sequencing of the V3–V4 regions in the bacterial 16S rRNA- gene produced a total of 7,744,670 reads (joined R1–R2 paired-end reads), with an average of 107,564 reads per sample (12 calves × 6 time points = 72 samples). After quality filtering, 1,438,378 sequences were removed, leaving 6,306,292 sequences for subsequent analyses (81.3% overall average retention rate, maximum 86%,

minimum 66.3%). Supplementary Table 1 reports the average retention rate and the number of sequences per treatment and time point: the number of sequences ranged from a minimum of 61,592 (±33,344) in the BM group at Wk1 to a maximum of 139,889 (±94,526) in the BM group at Wk4. The initial number of OTUs identified was 10,835; after filtering out OTUs with <10 counts in at least two samples, 3,264 distinct OTUs remained. Supplementary Figure 1 reports the sequence-based and sample-based rarefaction curves generated from the OTU table before filtering (10,835 OTUs), where the observed number of OTUs detected was plotted, respectively, as a function of the number of reads (up to 75,000) in each sample and of the number of samples. Both curves tend to plateau asymptotically, indicating that sequencing depth and the number of samples were adequate. Deeper sequencing or adding any other sample would not significantly increase the number of new OTUs discovered.

Alpha Diversity

Figure 2A illustrates the alpha (within-sample) diversity indices in the fecal microbiota of the two calf groups during the trial after correcting for baseline. Index values are averages per group, expressed as differences from values at baseline (Wk0). At Wk1, alpha diversity increased in both groups but slightly less in WM calves. At Wk2, all diversity indices increased in BM and decreased in WM. The difference between groups was further amplified at Wk4, two weeks after removing WM from the diet. The two groups reached similar levels at Wk6. At Wk8, the microbiota diversity decreased in both groups, but slightly more in BM. Figure 2B illustrates the significance values for all alpha diversity indices at all the experimental time points. At Wk4, the difference between WM and BM was statistically significant ($p < 0.05$) for all alpha diversity indices, indicating a substantial negative impact on the fecal microbiota diversity that persisted for at least two weeks after removing the antibiotic-containing WM from the diet. Equitability and Simpson evenness were significantly different at Wk1 and Wk2 ($p < 0.05$), respectively.

Beta Diversity

Figure 3 illustrates the first two dimensions from the (non-metric) multidimensional scaling of the Bray–Curtis dissimilarity matrix, clustering samples by treatment (top left), time point (bottom left), and treatment-and-time point (right). While the two groups (WM and BM) overlapped extensively, the fecal microbiota evolved by changing significantly during the first eight weeks of life ($p = 0.0069505$, from PERMANOVA between time points, 999 permutations). Concerning beta diversity between treatments at each time point, the BM and WM groups were separated at Wk4 (Figure 4, right), in line with the alpha diversity results (Figures 2A, B).

Impact of Waste Milk on the Fecal Microbiota Taxonomy

Figure 4 summarizes all the statistically significant taxonomy changes observed in the fecal microbiota. The changes occurring in WM calves compared to BM calves are illustrated in a heatmap as relative normalized OTU levels for each time point. Normalized OTU levels are detailed in Supplementary Figure 2, while significant values are illustrated in Supplementary Figure 3. As a general observation, and in agreement with the alpha diversity and beta diversity results, most differential taxa were less abundant in WM than in BM calves at all time points, except for the last time point, at Wk8.

Wk0 (Age: 3 Days)

At four days of life, the phylum Bacteroidetes was significantly more abundant in WM calves; this was reflected in the class Bacteroidia, order Bacteroidales, family Bacteroidaceae, and genus *Bacteroidetes*. The family Rikenellaceae with the related genus *Rikenellaceae* RC9 gut group and *Rothia* were also more abundant., the phylum Fusobacteria and the order Propionibacteriales with the genus *Propionibacterium* were less abundant, together with the family Bacillaceae and the genus *Acetatifactor*.

Wk1 (Age: 10 Days)

After one week of WM feeding, several taxa showed a significantly lower abundance in WM calves than in BM calves. These included the two classes Fusobacteria and Negativicutes, the two orders Fusobacteriales and Selenomonadales, the two families boneC3G7 and Veillonellaceae, and the seven genera [*Ruminococcus*] *gauvreauii* group, *Allisonella*, *Dialister*, *Megamonas*, *Megasphaera*, *Solobacterium*, and *Veillonella*. The order Rhodospirillales and the related family Rhodospirillaceae were more represented, together with Comamonadaceae. The three genera *Catenibacterium*, *Howardella*, and *Tyzzerella* were also higher.

Wk2 (Age: 17 Days)

After two weeks of WM feeding, numerous taxa were less abundant in WM vs. BM calves: the two phyla Bacteroidetes and Fusobacteria; the three classes including the related Bacteroidia and Fusobacteriia, together with Actinobacteria; the two related orders Bacteroidales and Fusobacteriales; the two families CFT112H7 and Prevotellaceae; and the seven genera *Acetatifactor*, *Fusicatenibacter*, *Lachnospiraceae* FE2018 group and UCG-005, *Prevotella* 7, *Prevotellaceae* NK3B31 group, and *Psychrobacter*. Only the genus *Tyzzerella* was higher in WM vs. BM calves.

Wk4 (Age: 31 Days)

The most significant differences between WM and BM calves were observed two weeks after the removal of WM from the diet, in line with the alpha diversity and beta diversity results. Numerous taxa were less abundant in WM calves, while only a few were more abundant. The most dramatic difference was seen for the phylum Firmicutes and the related class Clostridia, order Clostridiales, family Ruminococcaceae, and genera *Faecalibacterium* and *Ruminococcaceae* UCG-002. Less abundant were also the class Betaproteobacteria with the order Burkholderiales; the three families Alcaligenaceae, Lachnospiraceae, and Rikenellaceae; and the 11 genera *Alistipes*, *Christensenellaceae* R-7 group, *Erysipelotrichaceae* UCG-003, *Intestinimonas*, *Lachnospiraceae* NC2004 group, *Lachnospiraceae* NK4A136 group, *Lachnospiraceae* UCG-010, *Prevotellaceae* NK3B31 group, *Roseburia*, *Slackia*, and *Sutterella*. Only the family Enterococcaceae was higher in WM calves and the three genera, *Anaerovibrio*, *Mitsuokella*, and *Veillonella*.

Wk6 (Age: 45 Days)

Four weeks after removing WM from the diet, the family Fusobacteriaceae and the six genera *Catenibacterium*, *Faecalicoccus*, *Fusobacterium*, *Odoribacter*, *Shuttleworthia*, and *Solobacterium* were lower in WM vs. BM calves, the two genera *Anaerovibrio* and *Veillonella,* were higher.

Wk8 (Age: 59 Days)

Six weeks after removing WM from the diet, the abundance of several taxonomic groups was different in WM vs. BM calves. In contrast with all the previous time points, , most differential taxa were significantly higher in WM calves, as follows: the phylum Chlamydiae with the related class Chlamydiae, order Chlamydiales, family Chlamydiaceae, and genus *Chlamydophyla*, the family Campylobacteriaceae with the related genus *Campylobacter*, the family Lactobacillaceae with the related genus *Lactobacillus*, the family Pseudomonadaceae with the related genus *Pseudomonas*, together with the genera *Lachnoclostridium*, *Lachnospiraceae* NK4A136 group, *Sharpea*, and *Succiniclasticum*. Only the phylum Saccharibacteria and the genus *Lachnospiraceae* UCG-009 were less abundant in WM calves at this time point.

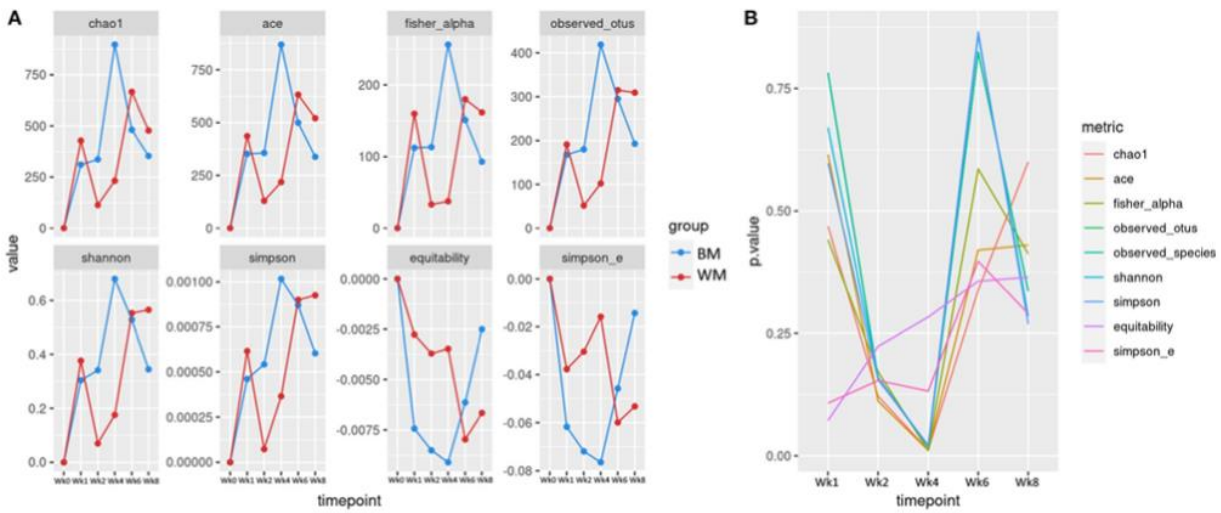

FIGURE 2 | (A) Alpha diversity indices at the different trial time points for the two groups: bulk tank milk (BM) and waste milk (WM). Index values are indicated as differences from baseline (Wk0 = 0). **(B)** Statistical significance of alpha diversity indices at the various time points.

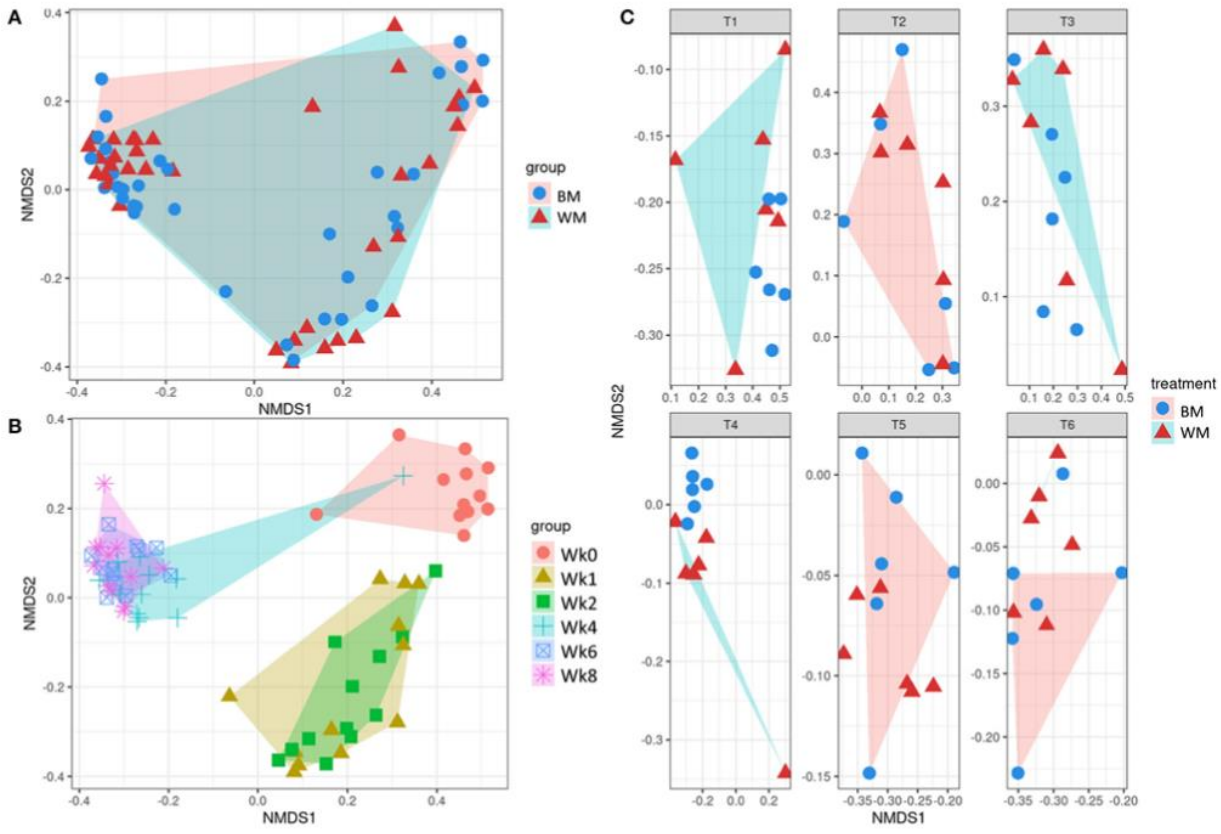

FIGURE 3 | Beta diversity according to treatment **(A)**, time point **(B)**, and treatment-by-time point **(C)**. The legends indicate the color codes and symbols used for the different sample groups [blue circles and pink shading, bulk tank milk (BM); red triangles and turquoise shading, waste milk (WM)] and time points (pink circle, Wk0; brown triangle, Wk1; green square, Wk2; turquoise cross, Wk4; blue box, Wk6; pink asterisk, Wk8).

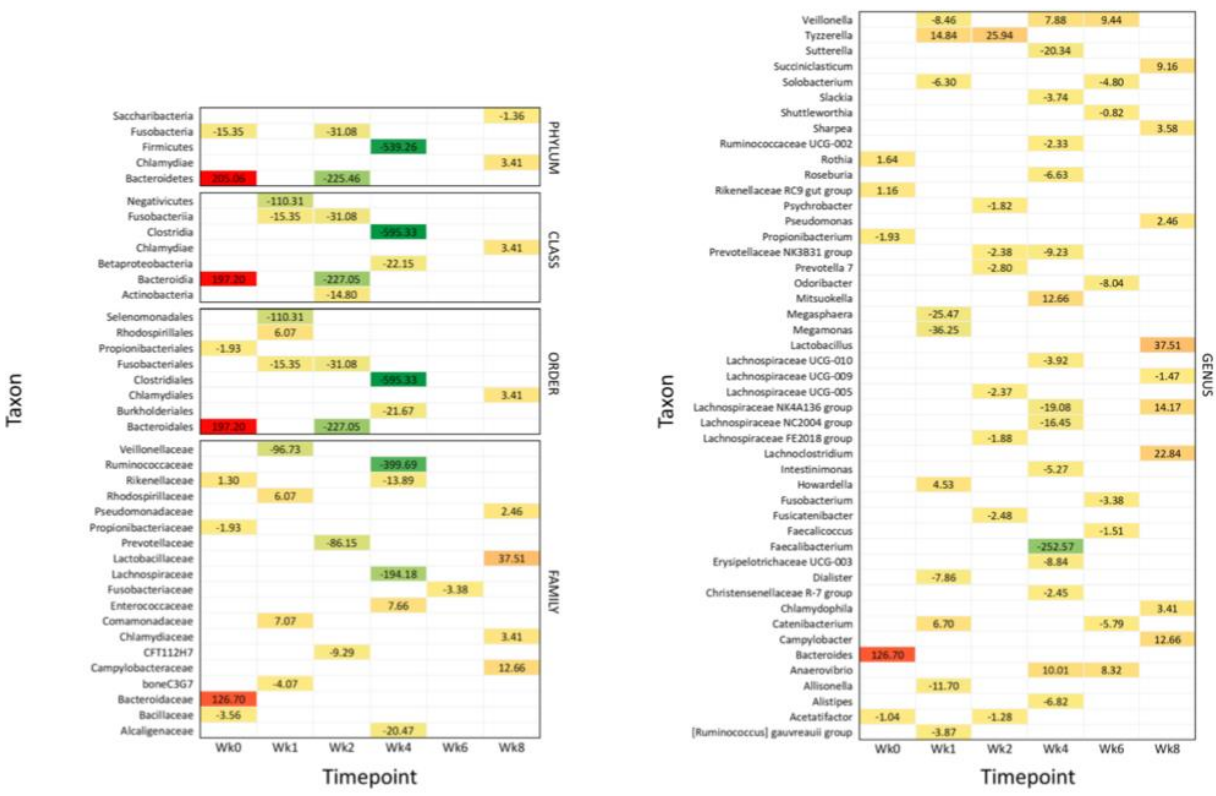

FIGURE 4 | Significantly different taxonomic groups in the fecal microbiota in the two calf groups at the different time points. The results are reported as a heatmap where red indicates a decrease and green indicates an increase of the normalized operational taxonomic unit (OTU) value in waste milk (WM) calves vs. bulk milk (BM) calves for each taxon at the different time points. The normalized OTU values and the statistical significance of the differences are reported in **Supplementary Figures 2**, **3**, respectively.

Discussion

Using WM for feeding calves seems a convenient perspective for the farmer for economic and practical issues, including its disposal, and because of its nutritional qualities. As highlighted by numerous researchers and reported in a recent European Food Safety Authority (EFSA) opinion paper, feeding calves with milk containing antibiotic residues presents a significant risk for developing antimicrobial resistance (Ricci et al., 2017). Another relevant issue is the action on the developing calf gut microbiome, with the potential reduction of overall diversity and the selective inhibition of antibiotic-sensitive microbial groups. Possible consequences are an increased susceptibility to intestinal diseases and the establishment of dysbiosis with adverse effects on animal health and welfare in later life (Ricci et al., 2017). Gut health results from multiple factors that maintain a disease-free status; in this respect, the gut microbiome is crucial (Bischoff, 2011). Dysbiosis, an imbalance in the gut microbiome, is associated with numerous gastrointestinal and autoimmune diseases (Videvall et al., 2020; Wu et al., 2012) and is typically characterized by a reduction in microbial diversity with the loss of beneficial microorganisms and the proliferation of pathobionts (Caruso et al., 2020; Petersen et al., 2014; Sekirov et al., 2010). The general principles governing resilience and dysbiosis seem to apply to most mammals (Marsilio et al., 2019; Sommer

et al., 2017; Winter et al., 2014), but further studies are required to unravel species-specific differences in consideration of the significant differences in the anatomy and physiology of digestion.

Study Strengths and Limitations

A relevant advantage of this study was the administration of standardized colostrum, TM, and WM, together with WM characterization in terms of antibiotic concentration and nutrient content. In this way, there were no differences in colostrum quality among calves or calf groups, and WM's composition and antibiotic content remained the same throughout the trial, some limitations were also present. For ethical and practical reasons, the number of calves enrolled in the trial was limited to six per group, and calves were enrolled sequentially, first in the BM and then in the WM group. The trial was conducted in a reduced time frame to offset these issues, and stringent statistics were applied to highlight the most relevant differences between the groups. We observed differences in BM and WM calves' fecal microbiota at the beginning of the trial. Newborn calves have an unstable microbiota, as on the first day of postnatal life, the microbial community's relative composition changes dramatically (Alipour et al., 2018), even minimal variations in the hour of sampling about the hour of birth may have led to this result. The dramatic changes occurring within 24 h from birth are followed by a relevant increase in the bacterial load, reducing the impact of the delivery time and reinforcing the reliability of the study findings. Another point to consider is that WM from cows with mastitis likely had a different milk microbiota in itself than BM. The different microbiota in calves fed with WM could have resulted from the microbes being ingested (or the ecological change these microbes created); the study design model used here did not allow us to dissect the effect of drug residues from other factors that differed between WM and BM, such as milk composition and milk microbiota effect on fecal microbiota (Addis et al., 2016). We cannot rule out a possible influence of the ORS on the WM calves' fecal microbiota (Omazic et al., 2013). The 16S rRNA gene analysis approach provides information only on bacteria. The gut microbiota also includes archaea, protozoa, viruses, algae, and fungi that play crucial roles in maintaining eubiosis (Berg et al., 2020; Marchesi et al., 2015). While bacterial communities mostly recover 30 days after heavy perturbations such as an antibacterial treatment, the fungal community may shift from mutualism toward competition (Seelbinder et al., 2020). Investigations by metagenomics or metaproteomics would also include the non-bacterial components of the calf hindgut microbiome and highlight possible functional profile alterations accompanying the taxonomy changes (Tanca et al., 2016; Pereira et al., 2018; Mao et al., 2015).

Results from 16S rRNA-gene sequencing may vary depending on the software (e.g., QIIME version) and the parameters used to process and analyze the data. The robustness of results to the Phred filtering threshold has been indicated (Biscarini et al., 2018), and more comprehensive sensitivity analyses of computer packages and parameters would shed light on these aspects. Our study was carried out on male calves for animal value issues and ethical aspects due to female calves' extended life expectancy. Long-term effects on the dairy farm are of interest mainly for what concerns female calves, and gender effects may have to be evaluated more carefully. The breed might also play a role in resilience to intestinal microbiota perturbations (Bergamaschi et al., 2020; Cremonesi et al., 2018). First-generation cephalosporins are widely used for the intra-mammary treatment of clinical mastitis and are, one of the antibiotic classes most likely to be found in WM from cows with bacterial mastitis (Redding et al., 2019; Tempini et al., 2018). The types and concentrations of antimicrobials on a farm can vary considerably according to management variables and time of year (Maynou et al., 2019). Some effects observed here might be antimicrobial-dependent, and other antibiotics in WM, broad-spectrum antibiotics or the same antibiotics at different concentrations may lead to different results (Raju et al., 2020). The pasteurization of WM might lead to different results by reducing the microbial load and removing the influence of the WM microbiome. However, pasteurization does not change the concentration of antibiotic residues significantly (Aust et al., 2013).

Impact on Calf Diarrhea Incidence and Weight Gain

During the two weeks of WM feeding, we observed a significant increase in calf diarrhea incidence. Mitigating pre-weaned calf mortality is a substantial challenge of the cattle industry, and enteric problems are among the major causes of newborn calf death (Malmuthuge et al., 2017; Uetake, 2013). When considering the limitations of prophylactic antimicrobial use (Smith, 2015), it is critical to minimize the factors that favor the onset of diarrhea and compromise pre-weaned calf gut health, including administering WM from mastitic cows. A related observation was the negative effect on calf growth. This reduced growth might lead to a slower beginning of the animal's productive life (Aghakeshmiri et al., 2017) and discourage WM use for feeding veal calves. Our results differ from those of previous reports on this topic. Aust et al. (Aust et al., 2013) observed that animals fed with WM had a similar growth rate to those fed with milk powder. This might be related to the very high incidence of diarrhea observed in our study in the first two weeks. The development of juvenile diarrhea is notoriously associated with reduced calf growth (Aghakeshmiri et al., 2017).

Alterations in Diversity and Taxonomy of the Microbiota at the Different Time Points

WM feeding led to a dramatic loss in the fecal microbiota's alpha diversity compared to BM. The difference was evident at Wk2 and highest at Wk4 concerning richness and uniformity. The adverse effects of WM in pre-weaned calves persisted and increased even under a diet with milk replacer containing probiotics integrated with pelleted starter feed, which should have led to an increase in the number of bacterial phylotypes in the calf gut (Malmuthuge et al., 2017). Increased microbiome diversity is associated with increased weight gain and a lower incidence of diarrhea in healthy calves at the fourth week of life (Malmuthuge et al., 2015, Oikonomou et al., 2013). Numerous taxa showed significant changes in abundance in calves fed with WM vs. BM, starting from the beginning of the trial and up to six weeks after removing WM from the diet. The significant differences observed in the fecal microbiota of WM calves might result from the selective action of cefalexin on some bacterial groups, with a resulting alteration in the microbial equilibria resulting in dysbiosis. Due to the elevated antibiotic concentration in WM, the significantly higher incidence of diarrhea in the first weeks of life could have been responsible for disrupting the microbial ecosystem and the consequent incomplete recovery of the healthy stable state (Sommer et al., 2017). At Wk1, *Veillonella* was already decreased in WM calves, in agreement with Van Vleck Pereira et al. (Pereira et al., 2016), who observed that *Veillonella* was the only genus significantly decreased in calves fed milk with drug residues at week 1. Their study, analyzed WM spiked with low amounts of antibiotics and assessed their effects only during WM feeding. In our study, after 2 and 4 weeks of removing WM from the diet, *Veillonella* increased compared to BM calves. This is undesirable since *Veillonella* produces toxic compounds by fermenting proteins and is negatively associated with short-chain fatty acid (SCFA) production and gut health (Brüssow, 2013). Also, at Wk1, the genus *Tyzzerella* was higher in WM than in BM calves. Previous studies in humans found a significant increase in *Tyzzerella* and *Tyzzerella 4* in Crohn's disease patients, indicating that this might be a negative occurrence (Olaisen et al., 2021). Another study demonstrated that this genus is overrepresented in patients with an unhealthy diet (Liu et al., 2019). Other beneficial taxa were decreased, such as *Megamonas* (Deng et al., 2017), which is also involved in producing SCFA. SCFAs are crucial for intestinal tissue metabolism and epithelium development and are absorbed into the bloodstream, providing energy for calf metabolism and growth (Amin et al., 2021). At Wk2, at the end of the WM feeding period, the Bacteroidetes phylum was significantly less abundant in WM than in BM calves. During the pre-weaning period, the rectal microbiota is composed mainly of Firmicutes and Bacteroidetes (Klein-Jöbstl et al., 2014); such a relevant change at this state indicates a strong impact of antibiotics on the microbial equilibria in the calf gut. This agrees with the observations of Maynou et al. (Maynou et al., 2019). In their study, most of the antimicrobials used to treat the cows from which WM originated

belonged to the β-lactam family and were mainly cephalosporins. Other studies did not observe disruptions at the phylum level (Pereira et al., 2016). This might be due to the higher antibiotic concentration in our WM. At Wk4, two weeks after removing WM from the diet, the phylum Firmicutes was dramatically lower in WM calves than in BM calves, and *Faecalibacterium* was the genus with the highest difference in abundance between the groups in the whole study. *Faecalibacterium prausnitzii*, the only known species in this genus, is strongly associated with positive effects on calf health and performance, including the reduction of diarrhea incidence and related mortality rate as well as increased weight gain (Foditsch et al., 2015), often together with *Roseburia* that was also less abundant in WM calves (Marques et al., 2016). These two bacteria are prototypical anti-inflammatory components of the gut microbiota and SCFA producers, especially butyrate, and *Faecalibacterium* represents one of the most abundant bacteria encountered in the feces of healthy animals (Foditsch et al., 2014). Calves with a higher abundance of *Faecalibacterium* at a very young age show higher daily weight gain and a lower incidence of diarrhea (Oikonomou et al., 2013). The whole Firmicutes phylum, mainly concerning the class Clostridia and the order Clostridiales, was dramatically less abundant in WM calves at Wk4. Dysbiosis is characterized by changes entailing a decreased prevalence of Clostridia (obligate anaerobes) (Antharam et al., 2013; Duvallet et al., 2017). Studies in mice showed that a lower relative abundance of Clostridia is associated with intestinal inflammation (Winter et al., 2014; Hildebrand et al., 2013). At Wk8, six weeks had passed since exposure to the cefalexin-containing WM, alpha diversity was higher for the first time in WM calves than in BM calves. This was accompanied by an increased carriage of taxa associated with veterinary and zoonotic diseases, including *Campylobacter*, *Chlamydophila*, and *Pseudomonas* (An et al., 2018; Klein et al., 2013; Kaltenboeck et al., 2005; Reinhold et al., 2008), with relevant consequences on calf health but also in terms of public health, as campylobacteriosis is the most important bacterial food-borne disease in the developed world (EFSA, 2014; Indikova et al., 2015). *Campylobacter* employs many survival strategies and can survive over an extended time in the ruminant gut (Indikova et al., 2015), and its association with *Pseudomonas* may further enhance its survival capabilities (Hilbert et al., 2010). In a general perspective, the increased presence of potential pathogens at the end of the trial, six weeks after exposure to the antibiotic-containing WM, may also suggest a status of failing resilience and reduced colonization resistance; the microbiota's competitive exclusion capacities (Sommer et al., 2017; Buffie et al., 2013). In the microbiota of WM calves was also more affected by the probiotics contained in the milk substitute, as they showed a significant increase in Enterococcaceae (Wk4, the only increased bacterial taxon above the genus at this time point) and Lactobacillaceae (Wk8, the most intense change observed in terms of increased taxa) two and six weeks after receiving WM with antibiotics, the WM calves' gut microbiome was

more susceptible to changes due to microorganisms administered with food; the gut microbiome of WM calves was less resilient. The phylum Saccharibacteria was one of the few taxa decreased in WM vs. BM calves at Wk8. Saccharibacteria, formerly known as TM7 (He et al., 2015;), increase in the mature rumen (Jami et al., 2013), are more abundant in older animals (O'Hara et al., 2020), and are part of the core rumen community in lactating dairy cows (Jami et al., 2012). This further suggests that feeding calves with antibiotic-containing WM may lead to long-term disruptions of gut microbiota physiology.

Conclusion

The microbiota plays a crucial role in the development and function of the gastrointestinal tract and gut health (Malmuthuge et al., 2017). It is essential for the proper development of the intestinal epithelium and the mucus layer (Lin et al., 2019; Sharma et al., 1995), the formation of lymphoid structures (Mebius et al., 2003), and the differentiation of immune cells (Petersen et al., 2014; Smith et al., 2011). Feeding pre-weaned calves with unpasteurized WM containing residual antibiotics might compromise these processes, impairing gut health and medium-term growth performances. The negative influences observed in the short term on alpha diversity, beta diversity, and taxonomy, together with the longer-term consequences on microbial taxa relevant for ruminal digestive processes and intestinal health, indicate that WM from cows treated with antibiotics should not be given to young calves.

Data availability statement

The data presented in the study are deposited in the EBI European Nucleotide Archive repository, accession number PRJEB42855.

Ethics statement

The animal study was reviewed and approved by the Institutional Committee for Animal Care of the University of Milan (protocol number 78_2018). Written informed consent was obtained from the owners for the participation of their animals in this study.

Author contributions

MP participated in the feeding trial, data analysis, data interpretation, and manuscript drafting. GS and AB contributed to the feeding trial, clinical monitoring of calves, sample collection, and clinical data analysis and interpretation. PC, BC, and FB contributed to the 16S data generation, analysis, and

visualization. VB contributed to the bacteriological culture of milk, the selection of cows, and data interpretation. PM and DP contributed to the study conception and design and data interpretation. MFA contributed to the study conception, design and coordination, data interpretation and visualization, and manuscript drafting. All authors contributed to the revision and approval of the final manuscript.

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Conflict of Interest

The authors declare that the research was conducted without any commercial or financial relationships that could be construed as a potential conflict of interest.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fvets. 2021.650150/full#supplementary-material

Supplementary File 1 | Metataxonomic pipeline command lines.

Supplementary Figure 1 | Rarefaction curves. The figures show the observed number of detected OTUs plotted as a function of the number of reads in each sample and the number of samples.

Supplementary Figure 2 | Normalized OTU values observed for all taxa showing statistically significant differences in abundance between WM and BM calves. The results are reported as a heatmap where red indicates the highest and green indicates the lowest normalized OTU value observed for each taxon at the different time points.

Supplementary Figure 3 | Statistical significance of the differences in normalized OTU abundances between WM and BM calves reported as a heatmap. The intensity of the red color increases with statistical significance.

Supplementary Table 1 | Average number of sequences per treatment and time point.

References

Addis MF, Tanca A, Uzzau S, Oikonomou G, Bicalho RC, Moroni P. (2016) The bovine milk microbiota: insights and perspectives from omics studies. Mol Biosyst. 12:1–29. doi: 10.1039/C6MB00217J

Aghakeshmiri F, Azizzadeh M, Farzaneh N, Gorjidooz M. (2017) Effects of neonatal diarrhea and other conditions on subsequent productive and reproductive performance of heifer calves. Vet Res Commun. 41:107–12. doi: 10.1007/s11259-017-9678-9

Alipour MJ, Jalanka J, Pessa-Morikawa T, Kokkonen T, Satokari R, Hynönen U, et al. (2018) The composition of the perinatal intestinal microbiota in cattle. Sci Rep. 8:10437. doi: 10.1038/s41598-018-31494-3

Amin N, Seifert J. (2021) Dynamic progression of the calf's microbiome and its influence on host health. Comput Struct Biotechnol J. 19:989–1001. doi: 10.1016/j.csbj.2021.01.035

An JU, Ho H, Kim J, Kim WH, Kim J, Lee S, et al. (2018) Dairy cattle, a potential reservoir of human campylobacteriosis: epidemiological and molecular characterization of Campylobacter jejuni from cattle farms. Front Microbiol. 9:3136. doi: 10.3389/fmicb.2018.03136

Anderson M. (2001) A new method for non-parametric multivariate analysis of variance. Austral Ecol. 26:32–46. doi: 10.1046/j.1442-9993.2001.01070.x

Andrews S. FastQC (2010) A Quality Control for High Throughput Sequence Data. Babraham Bioinforma

Antharam VC, Li EC, Ishmael A, Sharma A, Mai V, Rand KH, et al. (2013) Intestinal dysbiosis and depletion of butyrogenic bacteria in Clostridium difficile infection and nosocomial diarrhea. J Clin Microbiol. 51:2884–92. doi: 10.1128/JCM.00845-13

Aust V, Knappstein K, Kunz HJ, Kaspar H, Wallmann J, Kaske M. (2013) Feeding untreated and pasteurized waste milk and bulk milk to calves: effects on calf performance, health status and antibiotic resistance of faecal bacteria. J Anim Physiol Anim Nutr. 97:1091–103. doi: 10.1111/jpn.12019

Berg G, Rybakova D, Fischer D, Cernava T, Vergès MCC, Charles T, et al. (2020) Microbiome definition re-visited: old concepts and new challenges. Microbiome. 8:119. doi: 10.1186/s40168-020-00905-x

Bergamaschi M, Tiezzi F, Howard J, Huang YJ, Gray KA, Schillebeeckx C, et al. (2020) Gut microbiome composition differences among breeds impact feed efficiency in swine. Microbiome. 8:110. doi: 10.1186/s40168-020-00888-9

Biscarini F, Cozzi P, Orozco-ter Wengel P. (2018) Lessons learnt on the analysis of large sequence data in animal genomics. Anim Genet 49:147–58. doi: 10.1111/age.12655

Biscarini F, Cremonesi P, Castiglioni B, Stella A, Bronzo V, Locatelli C, et al. (2020) A randomized controlled trial of teat-sealant and antibiotic dry-cow treatments for mastitis prevention shows similar effect on the healthy milk microbiome. Front Vet Sci. 7:581. doi: 10.3389/fvets.2020.00581

Biscarini F, Palazzo F, Castellani F, Masetti G, Grotta L, Cichelli A, et al. (2018) Rumen microbiome in dairy calves fed copper and grape-pomace dietary supplementations: composition and predicted functional profile. PLoS ONE. 13:e0205670. doi: 10.1371/journal.pone.0205670

Bischoff SC. (2011) "Gut health": A new objective in medicine? BMC Med. 9:24. doi: 10.1186/1741-7015-9-24

Boccardo A, Biffani S, Belloli A, Biscarini F, Sala G, Pravettoni D. (2017) Risk factors associated with case fatality in 225 diarrhoeic calves: a retrospective study. Vet J. 228:38–40. doi: 10.1016/j.tvjl.2017.10.006

Bray JR, Curtis JT. (1957) An ordination of the upland forest communities of Southern Wisconsin. Ecol Monogr. 27:325–49. doi: 10.2307/1942268

Brunton LA, Duncan D, Coldham NG, Snow LC, Jones JR. (2012) A survey of antimicrobial usage on dairy farms and waste milk feeding practices in England and Wales. Vet Rec. 171:296. doi: 10.1136/vr.100924

Brüssow H. (2013) Microbiota and healthy ageing: observational and nutritional intervention studies. Microb Biotechnol. 6:326–34. doi: 10.1111/1751-7915.12048

Buffie CG, Pamer EG. (2013) Microbiota-mediated colonization resistance against intestinal pathogens. Nat Rev Immunol. 13:790–801. doi: 10.1038/nri3535

Caporaso JG, Kuczynski J, Stombaugh J, Bittinger K, Bushman FD, Costello EK, et al. (2010) QIIME allows analysis of high-throughput community sequencing data. Nat Methods. 7:335–6. doi: 10.1038/nmeth.f.303

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Lozupone CA, Turnbaugh PJ, et al. (2011) Global patterns of 16S rRNA diversity at a depth of millions of sequences per sample. Proc Natl Acad Sci USA. 108(Suppl):4516–22. doi: 10.1073/pnas.1000080107

Caruso R, Lo BC, Núñez G. (2020) Host–microbiota interactions in inflammatory bowel disease. Nat Rev Immunol. 20:411–26. doi: 10.1038/s41577-019-0268-7

Chao A, Lee SM. (1992) Estimating the number of classes via sample coverage. J Am Stat Assoc. 87:210–7. doi: 10.1080/01621459.1992.10475194

Chao A. (1984) Non-parametric estimation of the number of classes in a population. Scand J Stat. 11:265–70.

Chiesa LM, DeCastelli L, Nobile M, Martucci F, Mosconi G, Fontana M, et al. (2020) Analysis of antibiotic residues in raw bovine milk and their impact toward food safety and on milk starter cultures in cheese-making process. Lwt. 131:109783. doi: 10.1016/j.lwt.2020.109783

Cho I, Yamanishi S, Cox L, Methé BA, Zavadil J, Li K, et al. (2012) Antibiotics in early life alter the murine colonic microbiome and adiposity. Nature. 488:621–6. doi: 10.1038/nature11400

Constable PD. (2004) Antimicrobial use in the treatment of calf diarrhea. J Vet Intern Med. 18:8–17. doi: 10.1111/j.1939-1676.2004.tb00129.x

Cox LM, Yamanishi S, Sohn J, Alekseyenko AV, Leung JM, Cho I, et al. (2014) Altering the intestinal microbiota during a critical developmental window has lasting metabolic consequences. Cell. 158:705– 21. doi: 10.1016/j.cell.2014.05.052

Cremonesi P, Ceccarani C, Curone G, Severgnini M, Pollera C, Bronzo V, et al. (2018) Milk microbiome diversity and bacterial group prevalence in a comparison between healthy Holstein Friesian and Rendena cows. PLoS ONE. 13:e0205054. doi: 10.1371/journal.pone.0205054

Deng YF, Wang YJ, Zou Y, Azarfar A, Wei XL, Ji SK, et al. (2017) Influence of dairy by-product waste milk on the microbiomes of different gastrointestinal tract components in pre-weaned dairy calves. Sci Rep. 7:42689. doi: 10.1038/srep42689

Duvallet C, Gibbons SM, Gurry T, Irizarry RA, Alm EJ. (2017) Meta-analysis of gut microbiome studies identifies disease-specific and shared responses. Nat Commun. 8:1784. doi: 10.1038/s41467-017-01973-8

Edrington TS, Dowd SE, Farrow RF, Hagevoort GR, Callaway TR, Anderson RC, et al. (2012) Development of colonic microflora as assessed by pyrosequencing in dairy calves fed waste milk. J Dairy Sci. 95:4519– 25. doi: 10.3168/jds.2011-5119

Fisher RA, Corbet AS, Williams CB. (1943) The relation between the number of species and the number of individuals in a random sample of an animal population. J Anim Ecol. 12:42–58. doi: 10.2307/1411

Foditsch C, Pereira RVV, Ganda EK, Gomez MS, Marques EC, Santin T, et al. (2015) Oral Administration of Faecalibacterium prausnitzii decreased the incidence of severe diarrhea and related mortality rate and increased weight gain in preweaned dairy heifers. PLoS ONE. 10:e0145485. doi: 10.1371/journal.pone.0145485

Foditsch C, Santos TM, Teixeira AG, Pereira RV, Dias JM, Gaeta N, et al. (2014) Isolation and characterization of Faecalibacterium prausnitzii from calves and piglets. PLoS One. 9:e116465. doi: 10.1371/journal.pone.0116465

Greenwood C, Morrow AL, Lagomarcino AJ, Altaye M, Taft DH, Yu Z, et al. (2014) Early empiric antibiotic use in preterm infants is associated with lower bacterial diversity and higher relative abundance of Enterobacter. J Pediatr. 165:23–9. doi: 10.1016/j.jpeds.2014.01.010

He X, McLean JS, Edlund A, Yooseph S, Hall AP, Liu SY, et al. (2015) Cultivation of a human-associated TM7 phylotype reveals a reduced genome and epibiotic parasitic lifestyle. Proc Natl Acad Sci USA. 112:244–9. doi: 10.1073/pnas.1419038112

Heinrichs AJ, Rogers GW, Cooper JB. (1992) Predicting body weight and wither height in Holstein heifers using body measurements. J Dairy Sci. 75:3576–81. doi: 10.3168/jds.S0022-0302(92)78134-X

Hilbert F, Scherwitzel M, Paulsen P, Szostak MP. (2010) Survival of Campylobacter jejuni under conditions of atmospheric oxygen tension with the support of Pseudomonas spp. Appl Environ Microbiol. 76:5911–7. doi: 10.1128/AEM.01532-10

Hildebrand F, Nguyen TLA, Brinkman B, Yunta RG, Cauwe B, Vandenabeele P, et al. (2013) Inflammation-associated enterotypes, host genotype, cage and inter-individual effects drive gut microbiota variation in common laboratory mice. Genome Biol. 14:R4. doi: 10.1186/gb-2013-14-1-r4

Indikova I, Humphrey TJ, Hilbert F. (2015) Survival with a helping hand: campylobacter and microbiota. Front Microbiol. 6:1266. doi: 10.3389/fmicb.2015.01266

Jami E, Israel A, Kotser A, Mizrahi I. (2013) Exploring the bovine rumen bacterial community from birth to adulthood. ISME J. 7:1069–79. doi: 10.1038/ismej.2013.2

Jami E, Mizrahi I. (2012) Composition and similarity of bovine rumen microbiota across individual animals. PLoS ONE. 7:e33306. doi: 10.1371/journal.pone.0033306

John JA. SeqPrep (2011) v1.1—Tool for Stripping Adaptors and/or Merging Paired Reads With Overlap Into Single Reads

Kaltenboeck B, Hehnen HR, Vaglenov A. (2005) Bovine Chlamydophila spp. infection: do we underestimate the impact on fertility? Vet Res Commun. 29:1–15. doi: 10.1007/s11259-005-0832-4

Klein D, Alispahic M, Sofka D, Iwersen M, Drillich M, Hilbert F. (2013) Prevalence and risk factors for shedding of thermophilic campylobacter in calves with and without diarrhea in Austrian dairy herds. J Dairy Sci. 96:1203–10. doi: 10.3168/jds.2012-5987

Klein-Jöbstl D, Schornsteiner E, Mann E, Wagner M, Drillich M, Schmitz-Esser S. (2014) Pyrosequencing reveals diverse fecal microbiota in Simmental calves during early development. Front Microbiol. 5:622. doi: 10.3389/fmicb.2014.00622

Kogut MH, Arsenault RJ. (2016) Editorial: Gut health: the new paradigm in food animal production. Front Vet Sci. 3:71. doi: 10.3389/fvets.2016.00071

Kogut MH. (2019) The effect of microbiome modulation on the intestinal health of poultry. Anim Feed Sci Technol. 250:32–40. doi: 10.1016/j.anifeedsci.2018.10.008

Li W, Godzik A. (2006) Cd-hit: a fast program for clustering and comparing large sets of protein or nucleotide sequences. Bioinformatics. 22:1658–9. doi: 10.1093/bioinformatics/btl158

Lin L, Xie F, Sun D, Liu J, Zhu W, Mao S. (2019) Ruminal microbiome-host crosstalk stimulates the development of the ruminal epithelium in a lamb model. Microbiome. 7:83. doi: 10.1186/s40168-019-0701-y

Liu Y, Ajami NJ, El-Serag HB, Hair C, Graham DY, White DL, et al. (2019) Dietary quality and the colonic mucosa-associated gut microbiome in humans. Am J Clin Nutr. 110:701–12. doi: 10.1093/ajcn/nqz139

Malmuthuge N, Griebel PJ, Guan LL. (2015) The gut microbiome and its potential role in the development and function of newborn calf gastrointestinal tract. Front Vet Sci. 2:36. doi: 10.3389/fvets.2015.00036

Malmuthuge N, Guan LL. (2017) Understanding the gut microbiome of dairy calves: opportunities to improve early-life gut health 1. J Dairy Sci. 100:5996–6005. doi: 10.3168/jds.2016-12239

Mao L, Franke J. (2015) Symbiosis, dysbiosis, and rebiosis - the value of metaproteomics in human microbiome monitoring. Proteomics. 15:1142–51. doi: 10.1002/pmic.201400329

Marchesi JR, Ravel J. (2015) The vocabulary of microbiome research: a proposal. Microbiome. 3:31. doi: 10.1186/s40168-015-0094-5

Marques TM, Holster S, Wall R, König J, Brummer RJ, de Vos WM. (2016) Correlating the gut microbiome to health and disease. Gut-Brain Axis. 261–91. doi: 10.1016/B978-0-12-802304-4.00012-8

Marsilio S, Pilla R, Sarawichitr B, Chow B, Hill SL, Ackermann MR, et al. (2019) Characterization of the fecal microbiome in cats with inflammatory bowel disease or alimentary small cell lymphoma. Sci Rep. 9:19208. doi: 10.1038/s41598-019-55691-w

Maynou G, Chester-Jones H, Bach A, Terré M. (2019) Feeding pasteurized waste milk to preweaned dairy calves changes fecal and upper respiratory tract microbiota. Front Vet Sci. 6:159. doi: 10.3389/fvets.2019.00159

Mebius RE. (2003) Organogenesis of lymphoid tissues. Nat Rev Immunol. 3:292–303. doi: 10.1038/nri1054

Middleton JR, Fox LK, Pighetti G. Laboratory Handbook on Bovine Mastitis. (2017) New Prague, MN: National Mastitis Council, Madison, WI Available online at: http://nmconline.org/docs/lhb.htm (accessed May 4, 2015)

O'Hara E, Kenny DA, McGovern E, Byrne CJ, McCabe MS, Guan LL, et al. (2020) Investigating temporal microbial dynamics in the rumen of beef calves raised on two farms during early life. FEMS Microbiol Ecol. 96:fiz203. doi: 10.1093/femsec/fiz203

Oikonomou G, Teixeira AGV, Foditsch C, Bicalho ML, Machado VS, Bicalho RC. (2013) Fecal microbial diversity in pre-weaned dairy calves as described by pyrosequencing of metagenomic 16S rDNA. Associations of Faecalibacterium Species with Health and Growth. PLoS ONE. 8:e63157. doi: 10.1371/journal.pone.0063157

Olaisen M, Flatberg A, Granlund AB, Røyset ES, Martinsen TC, Sandvik AK, et al. (2021) Bacterial mucosa-associated microbiome in inflamed and proximal noninflamed ileum of patients with crohn's disease. Inflamm Bowel Dis. 27:12–24. doi: 10.1093/ibd/izaa107

Omazic AW, Tråvén M, Roos S, Mellgren E, Holtenius K. (2013) Oral rehydration solution with glycerol to dairy calves: effects on fluid balance, metabolism, and intestinal microbiota. Acta Agric Scand Sect A. 63:47–56. doi: 10.1080/09064702.2013.785585

Palleja A, Mikkelsen KH, Forslund SK, Kashani A, Allin KH, Nielsen T, et al. (2018) Recovery of gut microbiota of healthy adults following antibiotic exposure. Nat Microbiol. 3:1255–65. doi: 10.1038/s41564-018-0257-9

Paulson JN, Stine CO, Bravo HC, Pop M. (2013) Differential abundance analysis for microbial marker-gene surveys. Nat Methods. 10:1200–2. doi: 10.1038/nmeth.2658

Petersen C, Round JL. (2014) Defining dysbiosis and its influence on host immunity and disease. Cell Microbiol. 16:1024–33. doi: 10.1111/cmi.12308

Pravettoni D, Morandin N, Belloli A. (2014) Semeiologia Clinica Veterinaria. ed. P. Ciaramella Poletto Editore, Srl

Quast C, Pruesse E, Yilmaz P, Gerken J, Schweer T, Yarza P, et al. (2013) The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. Nucleic Acids Res. 41:D590–6. doi: 10.1093/nar/gks1219

R Core Team. (2018) R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria

Raju SC, Viljakainen H, Figueiredo RAO, Neuvonen PJ, Eriksson JG, Weiderpass E, et al. (2020) Antimicrobial drug use in the first decade of life influences saliva microbiota diversity and composition. Microbiome. 8:121. doi: 10.1186/s40168-020-00893-y

Redding LE, Bender J, Baker L. (2019) Quantification of antibiotic use on dairy farms in Pennsylvania. J Dairy Sci. 102:1494–507. doi: 10.3168/jds.2018-15224

Reinhold P, Jaeger J, Liebler-Tenorio E, Berndt A, Bachmann R, Schubert E, et al. (2008) Impact of latent infections with Chlamydophila species in young cattle. Vet J. 175:202–11. doi: 10.1016/j.tvjl.2007.01.004

Ricci A, Allende A, Bolton D, Chemaly M, Davies R, Fernández Escámez PS, et al. (2017) Risk for the development of Antimicrobial Resistance (AMR) due to feeding of calves with milk containing residues of antibiotics. EFSA J. 15:e04665. doi: 10.2903/j.efsa.2017.4665

Seelbinder B, Chen J, Brunke S, Vazquez-Uribe R, Santhaman R, Meyer AC, et al. (2020) Antibiotics create a shift from mutualism to competition in human gut communities with a longer-lasting impact on fungi than bacteria. Microbiome. 8:133. doi: 10.1186/s40168-020-00899-6

Sekirov I, Russell SL, Caetano M, Antunes L, Finlay BB. (2010) Gut microbiota in health and disease. Physiol Rev. 90:859–904. doi: 10.1152/physrev.00045.2009

Shannon C. (1948) A matematical theory of communication. Bell Syst Tech J. 27:379–423. doi: 10.1002/j.1538-7305.1948.tb01338.x

Sharma R, Schumacher U, Ronaasen V, Coates M. (1995) Rat intestinal mucosal responses to a microbial flora and different diets. Gut. 36:209–14. doi: 10.1136/gut.36.2.209

Simpson EH. (1949) Measurement of diversity. Nature. 163:688. doi: 10.1038/163688a0

Smith B, Wilson JB. (1996) A consumer's guide to evenness indices. Oikos. 76:70–82. doi: 10.2307/3545749

Smith G. (2015) Antimicrobial decision making for enteric diseases of cattle. Vet Clin North Am. 31:47–60. doi: 10.1016/j.cvfa.2014.11.004

Smith PM, Garrett WS. (2011) The gut microbiota and mucosal T cells. Front Microbiol. 2:111. doi: 10.3389/fmicb.2011.00111

Sommer F, Anderson JM, Bharti R, Raes J, Rosenstiel P. (2017) The resilience of the intestinal microbiota influences health and disease. Nat Rev Microbiol. 15:630–38. doi: 10.1038/nrmicro.2017.58

Tanaka S, Kobayashi T, Songjinda P, Tateyama A, Tsubouchi M, Kiyohara C, et al. (2009) Influence of antibiotic exposure in the early postnatal period on the development of intestinal microbiota. FEMS Immunol Med Microbiol. 56:80–7. doi: 10.1111/j.1574-695X.2009.00553.x

Tanca A, Fraumene C, Manghina V, Palomba A, Abbondio M, Deligios M, et al. .(2016) Diversity and functions of the sheep fecal microbiota: a multi-omic characterization. Microb Biotechnol 10:541–54. doi: 10.1111/1751-7915.12462

Tempini PN, Aly SS, Karle BM, Pereira RV. (2018) Multidrug residues and antimicrobial resistance patterns in waste milk from dairy farms in Central California. J Dairy Sci. 101:8110–22. doi: 10.3168/jds.2018-14398

Thames CH, Pruden A, James RE, Ray PP, Knowlton KF. (2012) Excretion of antibiotic resistance genes by dairy calves fed milk replacers with varying doses of antibiotics. Front Microbiol. 3:1–12. doi: 10.3389/fmicb.2012.00139

The European Union Summary Report on Trends and Sources of Zoonoses Zoonotic Agents and Food-borne Outbreaks in 2012. EFSA J. (2014) 12:3547. doi: 10.2903/j.efsa.2014.3547

Tyler JW, Hancock DD, Parish SM, Rea DE, Besser TE, Sanders SG, et al. (1996) Evaluation of 3 assays for failure of passive transfer in calves. J Vet Intern Med. 10:304–7. doi: 10.1111/j.1939-1676.1996.tb02067.x

Uetake K. (2013) Newborn calf welfare: a review focusing on mortality rates. Anim Sci J. 84:101–5. doi: 10.1111/asj.12019

Van Vleck Pereira R, Carroll LM, Lima S, Foditsch C, Siler JD, Bicalho RC, et al. (2018) Impacts of feeding preweaned calves milk containing drug residues on the functional profile of the fecal microbiota. Sci Rep. 8:554. doi: 10.1038/s41598-017-19021-2

Van Vleck Pereira R, Lima S, Siler JD, Foditsch C, Warnick LD, Bicalho RC. (2016) Ingestion of milk containing very low concentration of antimicrobials: longitudinal effect on fecal microbiota composition in preweaned calves. PLoS ONE. 11:e0147525. doi: 10.1371/journal.pone.0147525

Videvall E, Song SJ, Bensch HM, Strandh M, Engelbrecht A, Serfontein N, et al. (2020) Early-life gut dysbiosis linked to juvenile mortality in ostriches. Microbiome. 8:147. doi: 10.1186/s40168-020-00925-7

Wickham H. (2009) Ggplot2: Elegant Graphics for Data Analysis. Springer. doi: 10.1007/978-0-387-98141-3

Winter SE, Bäumler AJ. (2014) Why related bacterial species bloom simultaneously in the gut: principles underlying the “like will to like” concept. Cell Microbiol. 16:179–84. doi: 10.1111/cmi.12245

Wu HJ, Wu E. (2012) The role of gut microbiota in immune homeostasis and autoimmunity. Gut Microbes. 3:4–14. doi: 10.4161/gmic.19320

Yilmaz P, Parfrey LW, Yarza P, Gerken J, Pruesse E, Quast C, et al. (2014) The SILVA and “all-species Living Tree Project (LTP)” taxonomic frameworks. Nucleic Acids Res. 42:D643–8. doi: 10.1093/nar/gkt1209

Yousif MH, Li JH, Li ZQ, Maswayi Alugongo G, Ji SK, Li YX, et al. (2018) Low concentration of antibiotics modulates gut microbiota at different levels in pre-weaning dairy calves. Microorganisms. 6:118. doi: 10.3390/microorganisms6040118

Zou Y, Wang Y, Deng Y, Cao Z, Li S, Wang J. (2017) Effects of feeding untreated, pasteurized and acidified waste milk and bunk tank milk on the performance, serum metabolic profiles, immunity, and intestinal development in Holstein calves. J Anim Sci Biotechnol. 8:53. doi: 10.1186/s40104-017-0182-4

Appendix

Supplementary File 1. Metataxonomic pipeline command lines.

APPENDIX 1: METATAXONOMICS PIPELINE COMMAND LINES

To process 16S rRNA gene sequencing data, the following pipeline based on QIIME 1.9 was used [1]. The specific steps and parameters used are detailed below.

Joining paired-end reads

Paired-end reads were joined into single FASTQ files per sample:

```
multiple_join_paired_ends.py --input_dir=<sample_path> --output_dir=./ --include_input_dir_path --
parameter_fp=$PWD/qiime_parameters --read1_indicator _R1 --read2_indicator _R2
```

The method "SeqPrep" for the joining of paired-end reads (https://github.com/jstjohn/SeqPrep) was selected via the parameter file (qiime_parameters):

```
join_paired_ends:pe_join_method  SeqPrep
```

Quality filtering

Joined reads were then filtered for quality and saved into a unique FASTA file for all samples:

```
multiple_split_libraries_fastq.py --demultiplexing_method sampleid_by_file
--input_dir=<multiple_join_paired_ends/> --output_dir=./
--include_input_dir_path --remove_filepath_in_name
--parameter_fp=$PWD/qiime_parameters
```

Quality filter parameter were specified via the parameter file (qiime_parameters):

```
split_libraries_fastq:max_bad_run_length     3     >>     ./qiime_parameters
split_libraries_fastq:min_per_read_length_fraction          0.75          >>
./qiime_parameters      split_libraries_fastq:sequence_max_n       0      >>
./qiime_parameters   split_libraries_fastq:phred_quality_threshold   19   >>
./qiime_parameters
```

OTU picking

OTUs were determined by aligning quality-filtered reads against the QIIME-compatible SILVA reference FASTA file, release 123, with minimum 97% clustering (https://www.arb-silva.de/download/archive/qiime/):

```
pick_closed_reference_otus.py
--reference_fp SILVA123_QIIME/rep_set/rep_set_all/97/97_otus.fasta
--taxonomy_fp SILVA123_QIIME/taxonomy/taxonomy_all/97/raw_ taxonomy.txt
--parallel --jobs_to_start=32 --force
--input_fp=<multiple_split_library/>seqs.fna  --output_dir=./
```

Filter OTUs

OTUs were filtered by total count across samples greater than 10 distributed in at least 2 samples:

```
filter_otus_from_otu_table.py -i <closed_otupicking/>otu_table.biom -n 10 -s
2 -o ./otu_table_filtered.biom
```

Normalization of OTU counts

To account for uneven sequencing, OTU counts were normalized by cumulative sum scaling (CSS, [2]): normalize_table.py -i

```
<filter_otus/>otu_table_filtered.biom -a CSS -o CSS_normalized_otu_table.biom
```

Alpha diversity

Alpha diversity indexes were estimated from the filtered and normalized OTU table:

```
alpha_diversity.py -i <normalize_otu/>CSS_normalized_otu_table.biom
-m chao1,ace,fisher_alpha,observed_otus,shannon,simpson,equitability,simpson_e
-o ./alpha.txt -t SILVA123_QIIME/trees/97/97_otus.tre
```

Beta diversity

Beta diversity was estimated from the filtered and normalized OTU table:

```
beta_diversity.py -i <normalize_otu/>CSS_normalized_otu_table.biom -m
bray_curtis -o ./ -t SILVA123_QIIME/trees/97/97_otus.tre
```

Sequence-based rarefaction

To check whether sequencing depth was adequate, sequence-based rarefaction curves were generated from the unfiltered OTU table:

```
alpha_rarefaction.py -i <closed_otupicking/>otu_table.biom -m metadatamapping.csv
-o ./ --force --parameter=$PWD/qiime_parameters --parallel
--jobs_to_start=32 --max_rare_depth 75000 --min_rare_depth 100
```

Where metadatamapping.csv is the metadata file (feed supplementation treatments), and max_rare_depth is the median sequence counts per sample. Additional parameters were specified via the parameter file (qiime_parameters):

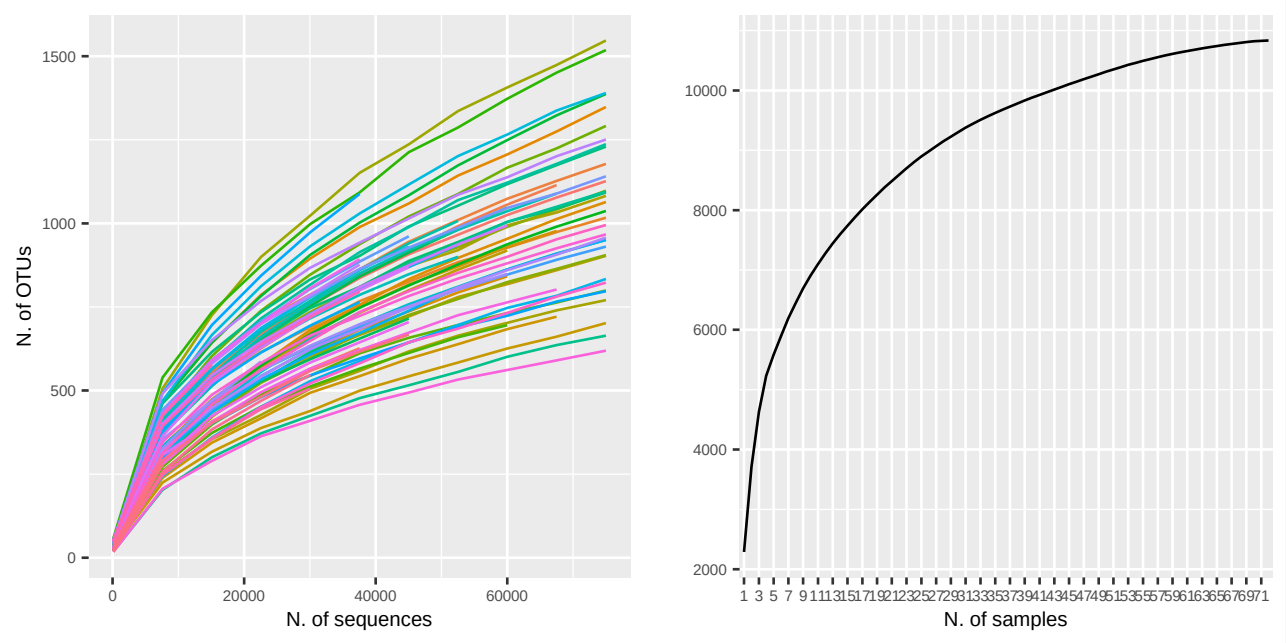
```
Alpha_diversity:metrics observed_otus,chao1,shannon,simpson,
goods_coverage,ace,fisher_alpha,equitability,simpson_e >> ./qiime_parameters
make_rarefaction_plots:resolution      800 >> ./qiime_parameters
```

References

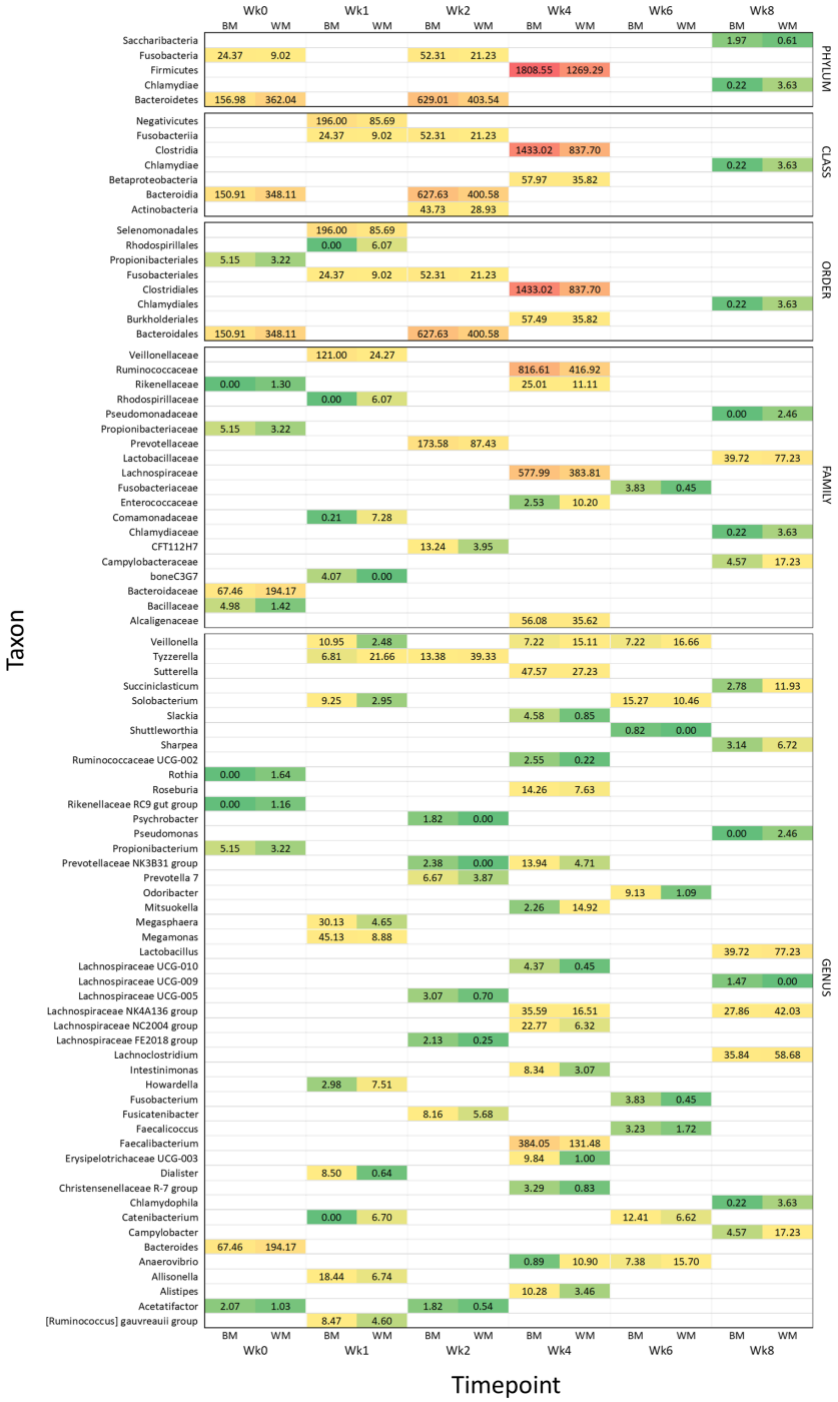
[1] Caporaso J, Kuczynski J, Stombaugh J, , others Bittinger, K,Caporaso, J G , Kuczynski, J , Stombaugh, J , Bittinger, K , Bushman, F D , Costello, E K , Fierer, N , Pen˜a, A G , Goodrich, J K , Gordon, J I , Huttley GA. QIIME allows analysis of high-throughput community sequencing data. Nature. 2010;7:335–336. doi:10.1038/nmeth.f.303.

[2] Paulson JN, Stine OC, Bravo HC, Pop M. Differential abundance analysis for microbial marker-gene surveys. Nature methods. 2013;10(12):1200–2. doi:10.1038/nmeth.2658.

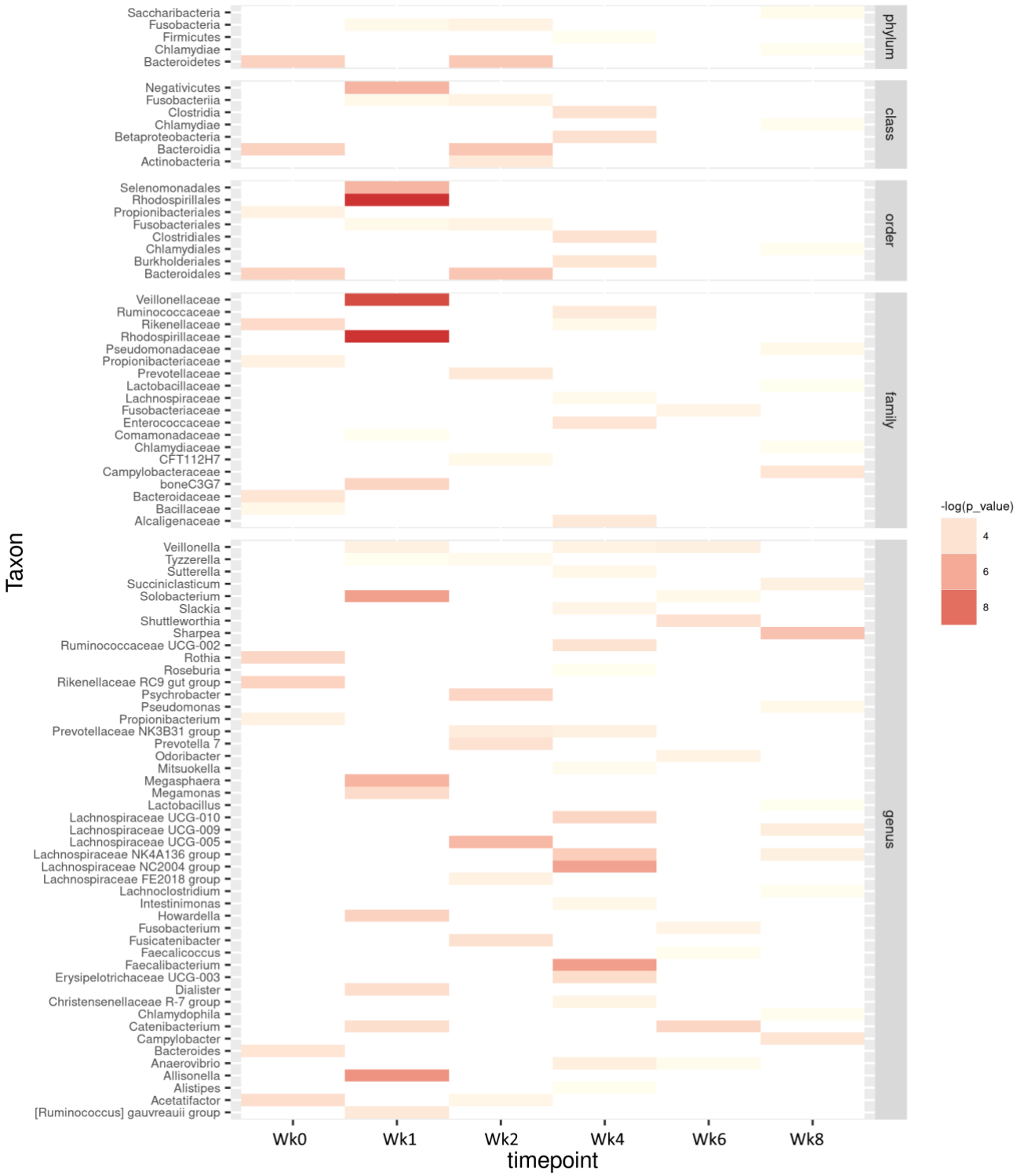
Supplementary Figure 1. Rarefaction curves. The figures show the observed number of detected OTUs plotted as a function of the number of reads in each sample and of the number of samples.



Supplementary Figure 2 | Normalized OTU values observed for all taxa showing statistically significant differences in abundance between WM and BM calves. The results are reported as a heatmap where red indicates the highest and green indicates the lowest normalized OTU value observed for each taxon at the different time points.



Supplementary Figure 3 | Statistical significance of the differences in normalized OTU abundances between WM and BM calves reported as a heatmap. The intensity of the red color increases with statistical significance.



Supplementary Table 1. Average number of sequences per treatment and time point.

Table_S1

	timepoint	N	NM	WM
n. sequences	T1	6	61592 +/-33344	74428 +/-43110
	T2	6	97989 +/-27237	76008 +/-27740
	T3	6	91951 +/-60080	73641 +/-41454
	T4	6	139889 +/-94526	76604 +/-49691
	T5	6	101748 +/-73122	80236 +/-29220
	T6	6	95741 +/-54542	81222 +/-31716
retention rate	T1	6	0,7989	0,8306
	T2	6	0,7807	0,7973
	T3	6	0,8085	0,8149
	T4	6	0,8277	0,8208
	T5	6	0,8222	0,8155
	T6	6	0,8151	0,8255

Conclusions

The first study about the application of MALDI-TOF MS in comparison with molecular methods, in our case *gap* PCR-RFLP, led us to provide more information comparing the two techniques. MALDI-TOF MS and *gap* PCR-RFLP provided comparable results for the most prevalent species of NASM and streptococci associated with sheep and goat mastitis. Our results suggest that when MALDI-TOF MS is not available, a reliable identification alternative can be offered by *gap* PCR-RFLP. Still, *Gap* gene sequencing may not efficiently resolve restriction profiles differing from the validated reference isolates. As for MALDI-TOF MS, integrating the spectrum library with more strains of the different species is recommended to improve identification performance further.

The second study survey provided a clearer picture of non-protein markers of mastitis. The Systematic Review work summarized the scientific literature on markers that can be measured by immunoassaybut not without difficulties. The first, and the most important, was the definition of appropriate keywords that would allow an extensive literature search without being too restrictive or, incurring an excessive overload of the dataset to be subsequently screened. For example, initially selected keywords included "ruminant" to limit the search to species of interest.

Scientific papers do not use the generic word "ruminant" but only the name of the dairy species investigated. It was necessary to eliminate the keyword to generate a more "inclusive" dataset and proceed with the subsequent elimination of scientific articles that dealt with other mammals (e.g., pigs, mouse models, or human medicine). The nature of the scientific papers themselves, which sometimes, even if they did relate to biomarkers of mastitis measured in milk by immunoassay, did not have the necessary keywords in either the title, abstract, or keywords to identify them as such. This required an additional stage of critical literature review by an experienced author. Although the PRISMA method and the careful definition of appropriate keywords enable the review work to be as objective as possible, it is helpful to include an experienced author on the working team. It also clearly illustrates how relevant it is to carefully choose the title, abstract, and keywords when writing a scientific article for effective indexing to allow easy identification by database searches.

The third study led to obtaining new data regarding the impact of feeding calves with WM from cows treated for mastitis with antibiotics on the balance of their gut microbiota. A large amount of discarded WM generates environmental pollution and represents the loss of a valuable resource that may serve as a good feed source for dairy calves because of its bulk quantities and high nutrient content andts use for raising dairy calves is widespread. Using WM for feeding calves is disputed, not only because of its potential pathogen load that may expose newborn calves to infectious diseases and harmful endotoxins but also because of the antimicrobial agent content. The constant antibiotic pressure

exerted on the calf microbiota by these residues may interfere with its physiological development by selectively inhibiting specific phylogenetic subgroups: it may increase the selection and transfer of antibiotic resistance genes to the gut microbiota. Despite the apparent economic advantages for the farmer, WM might impair the calf gut's correct physiological and immunological functionality and favor the selection of antibiotic-resistance traits. Data from the scientific literature and the results of our study indicate that the use of WM could have negative repercussions on the calf's intestinal health, the animal's growth, and the proper development of its microbiota, even though it is an economically attractive alternative for the farmer compared to disposal by other routes. There is also a real risk that this practice contributes to the selection and maintenance of antimicrobial-resistance traits in the bovine intestinal microbiota. The 2017 EFSA report on "Risk for the development of antimicrobial resistance (AMR) due to the feeding of calves with residues of antibiotics containing milk" lists some measures for the treatment of waste milk. According to the EFSA, producers should take appropriate measures to manage and dispose of waste milk in a way that minimizes its impact on the environment and public health. Producers can use various methods to treat waste milk on-farm, such as pasteurization or acidification. These treatments can reduce the pathogen load in the milk and make it safer for disposal. However, this is not useful for antibiotic residues. WM can be applied to agricultural land as a fertilizer, but this should be done in accordance with local regulations and best practices to avoid environmental contamination and public health risks. Producers can also contact a licensed waste disposal company to dispose of waste milk. This option can ensure that the milk is disposed of safely and in compliance with local regulations. The alteration of the pH can be also a helpful strategy but is negatively associated with the palatability of the milk and the risk is to change its properties. Finally, an alternative could be to degrade WM with enzymes, however, the risk is the possible abuse of enzymes by farmers. Overall, producers should follow best practices for waste milk management to reduce the potential risks associated with its disposal. They should also comply with local regulations and seek advice from relevant authorities when in doubt.

Future Perspectives

The first study led us to realize that MALDI-TOF mass spectrometry represents a very promising technique not only for the rapid identification of microorganisms but also for improving the identification of some of them. We would then analyze the NASM topic in detail in a prospective study to understand which species are most associated with subclinical and clinical mastitis to enable more conscious and informed management decisions in the future. We also intend to evaluate the use of MALDI-TOF mass spectrometry for the identification of, for example, Mycoplasma spp. to reduce the time and cost of diagnosis. To date, the most sensitive and specific diagnostic method for the identification of Mycoplasma spp. consists of a culture examination followed by PCR on the colony. This approach is effective but time-consuming and expensive. Therefore, since it is a fast-spreading pathogen, rapid identification is essential, and the MALDI-TOF mass spectrometer can be a useful tool to obtain reliable and rapid results. Over time, in addition to the rapid identification of causative agents of mastitis, rapid assessment of antibiotic resistance has also become increasingly important. For MALDI-TOF mass spectrometry software modules called "subtyping modules" have been implemented that also make it possible to rapidly screen certain antibiotic-resistant microorganisms, including Staphylococcus aureus MRSA. The module can have considerable utility in application to farm animals, as it provides an alert system for reporting suspected MRSA very quickly (a few minutes) and without requiring additional testing steps; the fact that many animals can be tested quickly and without additional cost can make it a useful sentinel system for the presence of MRSA on the farm. For these reasons, we intend to work on this topic to evaluate the applicability of this system. The second study allowed us to understand that diagnostic thresholds for the new markers, which have higher specificity and are still characterized by ease of detection, need to be better defined. For the third study, for calves that received WM in the pre-weaning period, it will be important to investigate in the future the presence of AMR-carrying bacteria, particularly the ESBLs at the intestinal level, also molecular characterization of genes encoding ESBLs is being planned. During the last phase of my Ph.D., I devoted myself to this aspect, working on a project evaluating the use of WM obtained from cows treated with antibiotics for the feeding of dairy calves in collaboration with the University of Bern at Swiss farms participating in a project to monitor antibiotic resistance in relation to on-farm practices. The objective is to evaluate the impact of feeding WM obtained from cows with mastitis on the prevalence of *E. coli* ESBL in the intestinal tract of calves. The project involves bacteriological analysis of calf feces samples for *E. coli* ESBL and characterization of resistance genes.