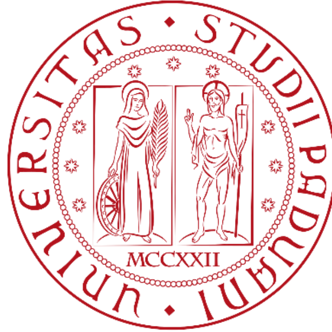

UNIVERSITÀ DEGLI STUDI DI PADOVA

DEPARTMENT OF LAND, ENVIRONMENT AGRICULTURE, AND FORESTRY

Second Cycle Degree (MSc) in Biotechnologies for Food Science

EFFECT OF SUBCLINICAL INTRAMAMMARY INFECTION ON THE MILK PROTEIN PROFILE AT THE QUARTER LEVEL IN HOLSTEIN CATTLE

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ABSTRACT

Milk and dairy products are essential agricultural sources of nutrition and milk production, depending on the consumption of dairy products, is increasing day by day with the increasing world population. Milk quality and production are of great interest in countries such as Italy, which is one of the world's most important players in the dairy industry and one of the largest producers of PDO cheeses. The bovine milk proteins establish the basis for a worldwide industry in dairy products, and as such have been intensively studied from back to nowadays. From these studies, we have a clearer awareness of the importance of these proteins in milk, milk quality, and bovine milk contains various ions, minerals, and vitamins that are essential for human health. One of the most important factors affecting milk quality is mastitis which is still one of the three main diseases that affect the profitability of the dairy industry, and accordingly, one of the other issues to be considered is udder health. Mastitis, which is one of the biggest economic problems in the dairy cattle industry from the past to the present, is the inflammation of the mammary gland. Mastitis is one of the most permanent and widely spread diseases in terms of importance to milk quality& and hygiene among dairy cattle worldwide.

The aim of this study of the existing relationship between subclinical mastitis and mammary gland inflammation status, and the detailed milk protein profile 450 lactating Holstein cows with the usage of RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography).

This study uncovered new insights into milk protein behavior in animals with subclinical IMI at the quarter level. The IMI mainly impacted casein fractions, particularly β-CN and αs1-CN proportions. The rise in SCC appeared to drive the most significant alterations, hinting that inflammation-linked proteolytic enzymes, rather than the infection itself, play a key role in changing milk protein. These findings reinforce SCC's significance as an indicator of udder health and milk quality.

Keywords: milk protein fractions, lactoferrin, subclinical mastitis, High-Performance Liquid Chromatography (HPLC)

RIASSUNTO

Il latte e i latticini sono fonti essenziali di nutrimento per l'agricoltura e la produzione di latte, a seconda del consumo di latticini, aumenta di giorno in giorno con l'aumento della popolazione mondiale. La qualità e la produzione del latte sono di grande interesse in paesi come l'Italia, che è uno dei più importanti attori mondiali nel settore lattiero-caseario e uno dei maggiori produttori di formaggi DOP. Le proteine del latte bovino costituiscono la base per un'industria mondiale dei prodotti lattiero-caseari e come tali sono state studiate intensamente fin dai giorni nostri. Da questi studi abbiamo una consapevolezza più chiara dell'importanza di queste proteine nel latte, della qualità del latte e il latte bovino contiene vari ioni, minerali e vitamine essenziali per la salute umana. Uno dei fattori più importanti che influenzano la qualità del latte è la mastite, che è ancora una delle tre principali malattie che incidono sulla redditività dell'industria lattiero-casearia e, di conseguenza, uno degli altri problemi da considerare è la salute della mammella. La mastite, che dal passato fino ai giorni nostri rappresenta uno dei maggiori problemi economici dell'industria delle vacche da latte, è l'infiammazione della ghiandola mammaria. La mastite è una delle malattie più permanenti e più diffuse in termini di importanza per la qualità e l'igiene del latte tra i bovini da latte in tutto il mondo. Lo scopo di questo studio è valutare la relazione esistente tra mastite subclinica e stato infiammatorio della ghiandola mammaria e il profilo dettagliato delle proteine del latte di 450 vacche Holstein in lattazione con l'utilizzo di RP-HPLC (cromatografia liquida ad alte prestazioni a fase inversa). Questo studio ha scoperto nuove informazioni sul comportamento delle proteine del latte negli animali con IMI subclinico a livello di trimestre. L'IMI ha avuto un impatto principalmente sulle frazioni di caseina, in particolare sulle proporzioni β-CN e αs1-CN. L'aumento dell'SCC sembrava causare le alterazioni più significative, suggerendo che gli enzimi proteolitici legati all'infiammazione, piuttosto che l'infezione stessa, svolgono un ruolo chiave nel cambiamento delle proteine del latte. Questi risultati rafforzano l'importanza dell'SCC come indicatore della salute della mammella e della qualità del latte.

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1. INTRODUCTION

1.1. DAIRY INDUSTRY

The world consumption of animal products (meat, dairy products, eggs, etc.), which are the most produced and valuable, rich in protein, and agricultural products, has increased in recent decades by increased populations and incomes (FedericoFroldi, 2022). Milk and dairy products are essential agricultural sources of nutrition and the dairy products connected to milk production (81% cow milk, 15 % buffalo milk, and 4 % for goat, sheep, and camel milk composite) increased by approximately 1.1 % to 887 Mt in 2021 more than the other agricultural products Dairy product consumption is expected to rise until 2031 in consideration of this (FAO, 2022) (ISMEA, 2021).

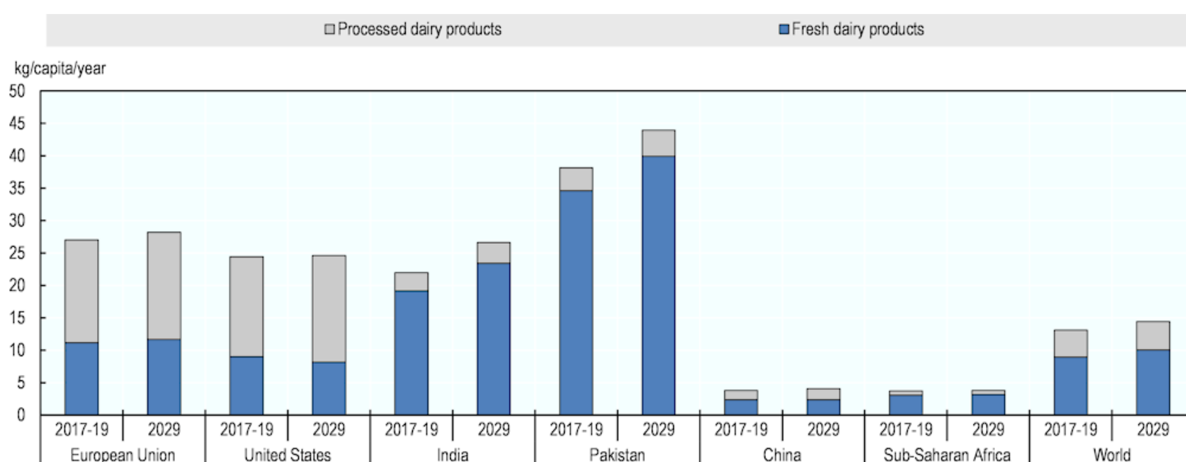


Figure 1. Per capita consumption of processed and fresh dairy products in milk solids. (OECD/FAO, 2020).

Note: Milk solids are calculated by adding the amount of fat and non-fat solids for each product; processed dairy products include butter cheese, skim milk powder, and whole milk powder.

In Italy, dairy is of great importance, with 30 % of the total agricultural area in 2018; This production, which is more than 12,744,000 tons of milk per year, makes Italy one of the world's most important players in the dairy industry and one of the largest producers of PDO cheeses. According to CLAL's data, 34.2 % of milk is delivered to the EU and 5.9 % of dairy exports come from Italy (the year 2021) (CLAL, 2021), (FAOSTAT, 2021a). Cow milk is produced in many different regions of Italy, especially in, Lombardia, Emilia-Romagna and Veneto, which account for 69 % of total milk production, but also in Piemonte, Trentino-Alto Adige, Lazio, Puglia, and Friuli-Venezia Giulia (Sabia et al., 2018).

1.2. MILK COMPOSITION

From a biological point of view, milk is the product of the mammary glands of mammals, including humans. Milk is a product of secretion and excretion of the mammary gland as it contains both synthesis and filtration substances. Milk production in cows starts after giving birth and normally continues for ten months; the milking duration depends on many factors, especially the health and nutrition of the animal. The primary constituents of milk are, in descending order: - water (87.5 %); milk solids (12.4 %) - carbohydrates, mainly including lactose (4.6 %); - lipids (4.2 %), mainly including triglycerides, collected in lipid globules; - proteins (3.4 %) are divided in caseins (organized in micelles) and whey proteins; - mineral salts; - enzymes, vitamins and trace elements (Taylor&Kabourek, 2003).

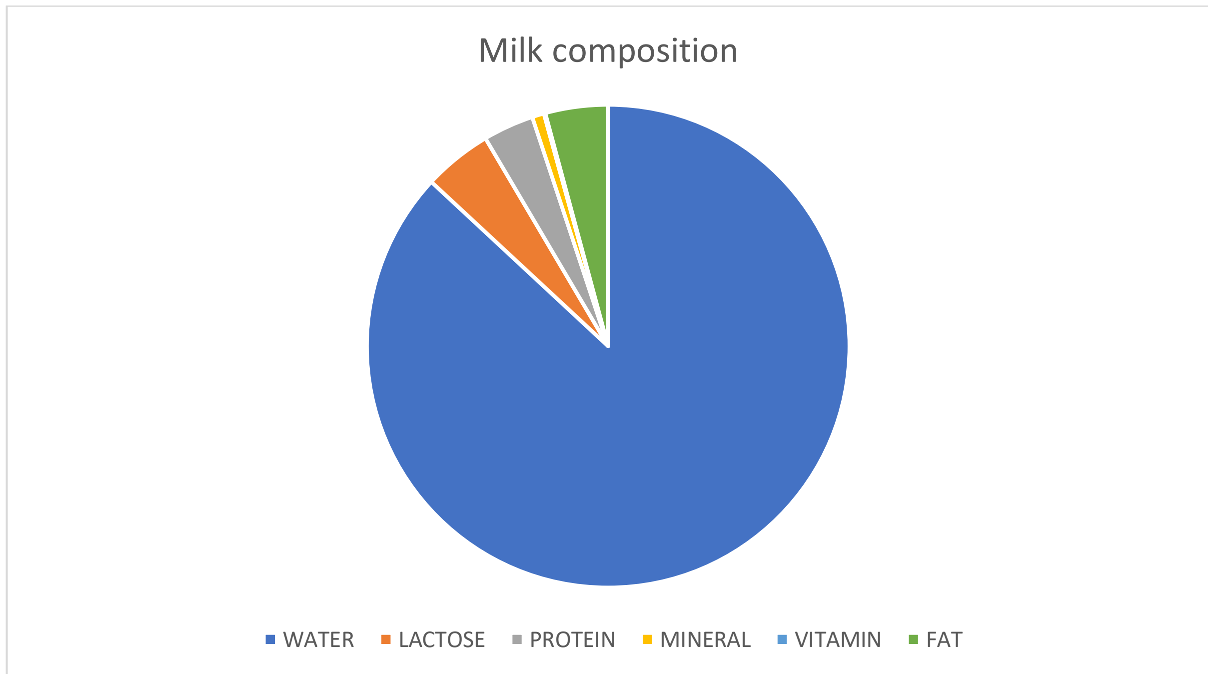


Figure 2. Cow's Milk Composition, referred to as ash; (Water (87 %), Lactose (4.6 %), Fat(4.2 %) Protein(3.4 %), Mineral(0.8 %), Vitamin(0.1 %). (T.H., 2022).

1.2.1. MILK PROTEIN

The bovine milk proteins establish the basis for a worldwide industry in dairy products, and as such have been intensively studied from back to nowadays. From these studies, we have a clearer awareness of the importance of these proteins in milk, milk quality, and bovine milk contains various ions, minerals, and vitamins that are essential for human health.

Figure 3 depicts the six primary milk proteins found in cattle. These genes, which are highly polymorphic and characterized by nonsynonymous-synonymous mutations, code for the casein (CN), -Lactoglobulin (-La), and -Lactalbumin (-La) proteins, of which more than 60 protein variants have been discovered thus far. At the same time, milk protein variants are invaluable for breed characterization, phylogenetic studies, and diversity.

On the other hand, it also has a place in human nutrition, for example, the production of milk with nutritional qualities, namely hypoallergenic milk. (Martin et al., 2002), (Bonfatti et al., 2008). There are many methods for analyzing milk protein fractions. These are electrophoretic techniques and isoelectric focusing (IEF), high-performance liquid chromatography (HPLC) by ion exchange, hydrophobic interactions and reversed-phase methods, capillary electrophoresis and capillary zone electrophoresis, mass spectrometry.

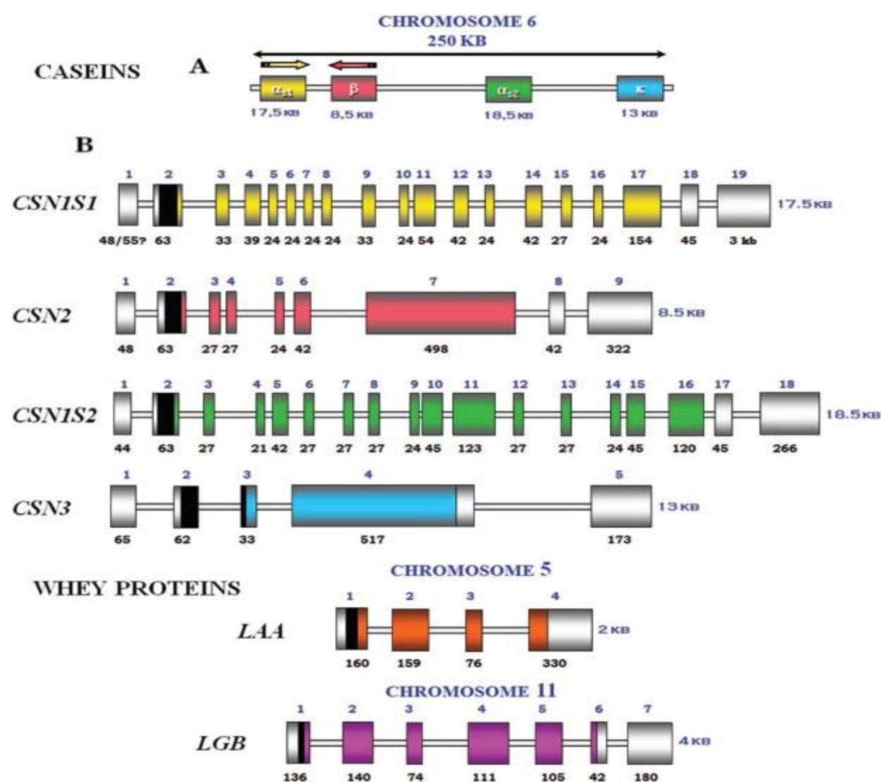


Figure 3. Structural organization of the transcription units encoding the 6 main milk proteins. (Martin et al., 2002). Generally, proteins contained in ruminant milk are coded by 6 structural genes. The 4 casein genes are linked in a 250-kb cluster mapped on chromosome 6. In physical order, these genes are CSN1S1, CSN2, CSN1S2, and CSN3 and encode αs1-CN, β-CN, αs2-CN, and κ-CN, respectively. This gene cluster is also referred to as the CN locus or super locus. The 2 main whey proteins, α-LA and β-LG, are coded by LAA and LGB genes, mapped on chromosomes 5 and 11, respectively.

In this study, the RP-HPLC method was applied for the simultaneous qualitative and quantitative analysis of bovine caseins and whey proteins, which allows rapid and automated analysis characterized by good separations, high resolutions, and accuracy and reproducible results.

1.2.1.1. CASEINS (α, β, κ)

Caseins are the major protein component of milk, functioning as nutritive carriers of both amino acids and minerals in milk. They represent approximately 80 % of the nitrogenous substances in bovine milk and are a family of phosphoproteins synthesized in the mammary gland and secreted in large colloidal aggregates called micelles responsible for the unique physical properties of milk. Their relatively high charge and hydrophilic properties distinguish them from many other proteins. Their abundance and importance in the dairy industry have made them one of the most studied dairy proteins as they are directly involved in curd formation and cheese yield (Glantz et al., 2010), (Bonfatti et al., 2008). They are divided into different fractions having different molecular weights and different affinity for water: α_{s1}, α_{s2}, β, and κ. The quantity and proportion of these proteins depend on several factors, among which some of the most important are genetic variants and breed (Ginger &Grigor, 1999). The biggest difference between α, β, κ caseins is the phosphate group per mole of casein, therefore their structures are different as seen in figure 4.

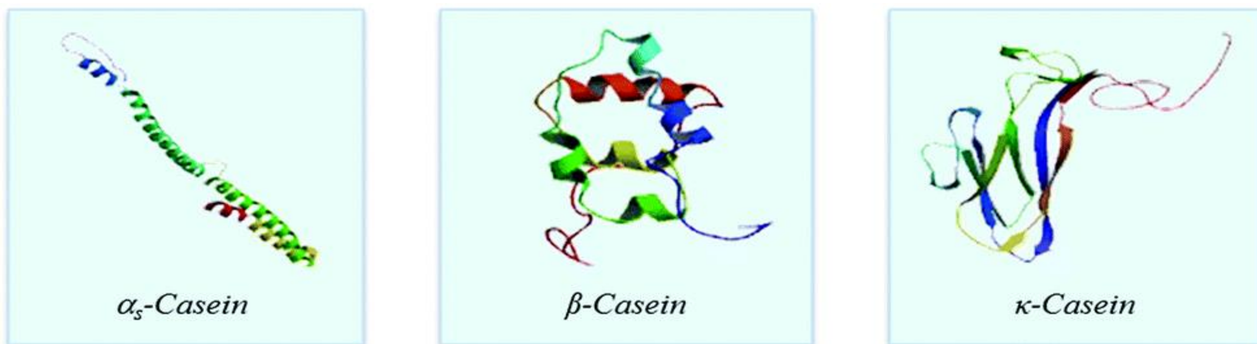


Figure 4. Structure of caseins.

They occur in milk in the form of an organic and mineral complex, the micelle, which consists of spherical particles called sub-micelles. In the casein micelle, the sub-micelles are held together and stabilized by ionic bonds with calcium phosphate and by hydrophobic interactions via phosphate groups, and the different casein fractions are linked with organic bonds of various kinds inside the sub-micelle. The important impact on the milk is the smaller the size of the micelles, the greater their stability and therefore consistency and casein coagulum yield (Manuelian et al., 2018).

α_{s1}-casein (α_{s1}-CN), accounts for approximately 35-38 % of the casein micellandes, is the main and most abundant protein fraction in bovine milk. It's highly phosphorylated and bovine milk consists of four isoforms containing 10 to 13 phosphates. There are five genetic variants, of which variant B is the most common in cattle breeds and tends to clump in the presence of calcium. (Penasa et al., 2012).

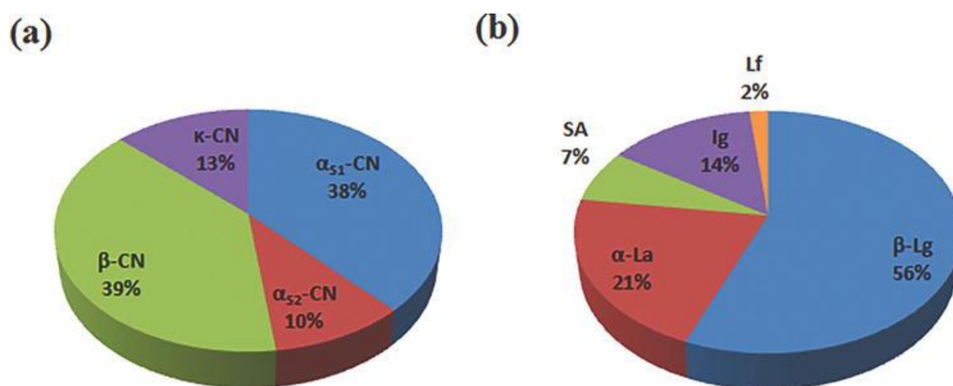


Figure 5. Proportions of the different caseins (a) and whey proteins (b) in cow's milk.(abbreviations: β-CN: β-casein; α_{s1}-CN: α_{s1}-casein; α_{s2}-CN: α_{s2}-casein; κ-CN: κ-casein; β-Lg: β-lactoglobulin; α-La: α-lactalbumin; SA: serum albumin; Ig: immunoglobulins; Lf: lactoferrin) (Chaiyabutr, 2022).

α_{s2}-casein (α_{s2}-CN) accounts for approximately 10 % of the total casein proportion and is the most hydrophilic of the casein with three clusters of anionic phosphoryl and glutamyl residues in the structure.

β-casein (β-CN), is the second most abundant milk protein and and it has 209 amino acid residues in its protein chain. It constitutes approximately 35-39 % of the total casein in bovine milk. It is found in twelve genetic variants (A1, A2, A3, B, C, D, E, F, G, H1, H2, I), the most common variants are A1-A2. The protein binds to calcium at its phosphorylated regions. The calcium then binds the caseins together and forms micelles. Beta-casein has a significant role in the determination of the surface properties of the micelles (Cieślińska et al., 2019), (Caroli et al., 2016).

κ-casein (κ-CN); in bovine milk, it represents about 12-13 % of the total caseins and is found in five genetic variants (A, B, B2, C, and E) among which the most common are A and B. Casein micelle diameters in milk samples with the κ-casein variants AB, BB, or BC are less than 200 nm, in contrast to those in milk samples with the variations AA, AC, and AE, which had micelle diameters above 200 nm (Panthi&Ram, 2017). κ-casein is very important in cheese production: it plays a major role in milk coagulation and, accordingly, in the formation of the desired texture. When the pH is below 4.6 or at high temperature, it binds to denatured whey proteins, mainly β-lactoglobulin.

1.2.1.2. WHEY PROTEINS

Whey proteins are combination of proteins with various functional properties, and they consist about 20 % of milk proteins. The primary ones are β-lactoglobulins (β-LA: 56-58 %), α-lactalbumins (α-LA; 21 %), immunoglobulins (14 %), bovine serum albumin (BSA; 5-7 %), protease-peptones, and enzymes. They are rich in essential amino acids, especially sulfur amino acids, and therefore have a high biological value, making them particularly interesting for the formulation of supplements for human use (H.M.C.De Azeredo, 2014). Whey proteins are found in solution in milk and are drained with the whey after casein coagulation during cheese production.

β-Lactoglobulin (β-LG) is the main and typical globular whey protein in bovine milk approximately 10 %–15 % of total milk proteins and 56-58 % of whey protein, in addition to this, β-Lactoglobulin eight genetic variants are known now (A, B, C, D, E, F, G, and Dr).

The biological function of bovine β -lactoglobulin, which is not found in human milk, is unclear, but it exhibits strong binding affinities for fatty acids, phospholipids, and aromatic compounds. In addition, it is important from a nutritional point of view, considering its high biological value. Some studies report a positive effect of the β-LG B allele on curd coagulation time, which could be due to lower β-LG production and therefore to an increase in the total casein content (Fox&Lowe, 2021), (Fenelon et al., 2019).

α-Lactalbumin (α-LA) is the second most important protein after β-Lactoglobulin and has a molecular weight of 14 kDa. The presence of four intramolecular disulfide bonds and the binding of calcium balances the structure of α-LA. It is an important source of energy and is small, globular protein rich in cysteine that is necessary as a coenzyme for the biosynthesis of lactose, and it is found in the three genetic variants (A, B and C) (Fuquay, 2011). One of the sources of bioactive peptides and essential amino acids, α-LA is a component of lactose synthesis necessary for lactose creation and it plays a significant role in the manufacture of milk (Layman et al., 2018), (O.L. Ramos, 2016).

Bovine serum albumin (BSA) constituting 5-7 % of whey proteins, significantly impacts a major effect on the health of cows' udders according to physico-chemical properties. According to specific research, the amount of bovine serum albumin in milk has a relationship between mammary gland health and somatic cell count (SCC) (Lieske et al., 2005).

Bovine immunoglobulins (Ig) are high molecular weight glycoproteins with immune properties, they can bind to bacteria and spores as well as to many allergens. They are divided into IgA, IgM, and IgG (IgG1 and IgG2); IgA has a more pathogenic binding capacity and greater stability against proteolytic degradation than IgG. They are synthesized by B lymphocytes and reach the mammary gland via the blood.

Protease peptones: some of which are specific to milk, are a heterogeneous mixture of peptides derived from βeta-casein proteolysis (Goulding et al., 2020).

Lactoferrin (LF) is an iron-binding glycoprotein found in many mammalian milks. The amount of lactoferrin varies considerably between species. For example, while it is found in low amounts in bovine milk (0.5 mg/L in mature milk), it is the most abundant whey protein in human milk (1 g/L in mature milk) after α-lactalbumin (Yang et al., 2018) (Recio et al., 2014).

The nutritional and functional role of lactoferrin is not yet fully understood, however, it is known that it is used to develop immunity, resistance to infection, control of non-communicable diseases, iron absorption, and human health in general in addition some studies show that lactoferrin related in various physiological and protective actions, among which some of the most studied to date are antioxidant, anti-tumor, anti-inflammatory, and antimicrobial activities (Superti, 2020).

In addition, about 70 natural (native) enzymes have been identified in studies on milk, such as lactoperoxidase, catalase, xanthine oxidase, proteases, lipases, etc. Each enzyme has different properties on milk, for example, some of them have an important antibacterial function (lactoferrin, lactoperoxidase, lysozyme), while others attack the constituents of the milk and can therefore determine variations in the technological characteristics of the same (lipase, protease) (Fox& Kelly, 2006).

1.3. ANATOMY & PHYSIOLOGY OF MAMMARY GLAND

The number, shape, and topography of the mammary gland are characteristic of different animal species. In each mammary gland (Figure 6) it is possible to distinguish a part called the mammary body and the nipple on which there are lactiferous pores from which the milk comes out. The number of milk pores varies according to the species. The mammary gland of the cow is divided into four quarters completely separated from each other by a septum of connective tissue. Although anastomoses (connections between structures) of the blood system from one quarter to another and between the two hemi parts have been demonstrated, each quarter is a separate gland. (Nickerson&Akers, 2011).

If we follow the orifice present at the apex of the nipple, we note that this leads to a short papillary duct which is kept closed by two sphincters made up of smooth muscles. The papillary duct leads into a cavity that practically occupies the entire nipple, which is called the nipple cistern. This in turn communicates extensively with the glandular cistern which is located at the base of the nipple and

has an irregular shape as numerous lactiferous ducts open in it, which gives the complex an almost spongy appearance. Following the lactiferous ducts, they penetrate deeply into the mammary tissue and repeatedly divide into smaller and smaller ducts. At the branching points, there is usually a narrowing of the lumen. The ducts that have the smallest dimensions are called terminal ducts and lead into small evaginations which constitute the mammary alveoli. These have a diameter of 0.1-0.4 mm and are lined with milk secretory cells. The alveoli are covered by a thin layer of secretory cells, which in turn are surrounded by the so-called myoepithelial cells which are provided with numerous ramifications constituting a sort of basket around the alveolus and which are also present along the ducts in, however, they are arranged roughly parallel to the axis of the ducts themselves. Although the milk secreted by the different species may be significantly different, the structure of the mammary alveolar cell, which represents the basic unit of milky secretion, is quite similar (Banerjee et al., 2022). In it both the endoplasmic reticulum and the Golgi apparatus are particularly developed; lysosomes are scarce, ribosomes are very abundant, and secretory vesicles are also present, which contain especially casein, and lipid globules information. However, the appearance of the mammary cells varies depending on whether the mammary is at rest, pregnant, or active: in the latter case, it shows considerable differences depending on whether the alveolus is empty or full of milk (Biswas et al., 2022).

1.4. MASTITIS

Mastitis, one of the most important economic problems to be considered in the dairy cattle industry from the past to the present, is the mammary inflammation gland (Figure 6) and is usually caused by different agents (i.e. *Staphylococcus aureus, Staphylococcus epidermidis, Mycoplasma* spp. and *Corynebacterium bovis*) (Ratcliffe et al., 2008). The pathogen (including viruses, bacteria, fungi, and their toxins) generally inserts through the udder end and maintains to reach the mammary gland inside the udder, then begins increasing and producing toxins that evoke the immune responses in the animal to fight the infections, which cause symptoms of mastitis: milk coagulation, decrease in the synthesis

of the main components of milk; such as lactose, fat, non-fat solids and casein, fever, swelling of the udder, decreased dry matter intake, color changes, increases somatic cell count in milk, water loss, and death. Additionally, mammary gland health disorders and mastitis can cause considerable losses in milk yield, alterations in its quality, fertility disorders, and even systemic diseases (Southern Agricultural Research Institute, 2022).

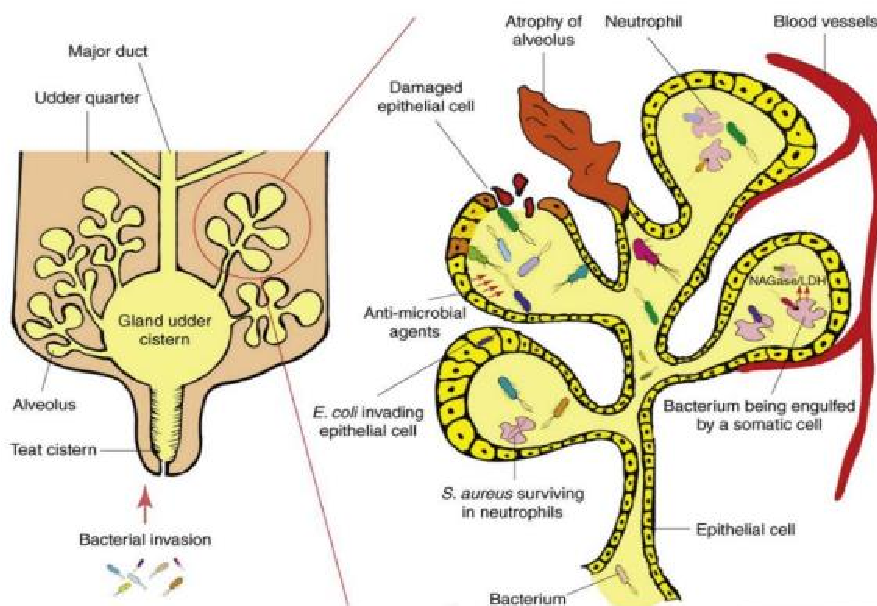


Figure 6. Schematic representation of mastitis development in an infected udder(A. Iyer, 2014).

In the main, bovine mastitis, the most significant disease of the dairy industry, has remarkable effects on farm and country economics due to a reduction in milk production, zoonotic effect, and treatment costs. There are many tests and methods used in the diagnosis of mastitis. Firstly, a cow's udder and milk can be physically controlled. If the cow has a red, hard, or warm udder or clots, flakes, blood, or watery secretion observed during milking, it may have mastitis. Besides the physical examination, there are many tests used to diagnose mastitis such as the bacteriological analysis, which is the gold standard, the CMT (California mastitis test), Somatic cell counts (SSC), Catalase or pH test, Strip cup test, Bromothymol blue test, etc. (Mohan, 2019).

1.4.1. TYPES OF MASTITIS

Mastitis is a prevalent and economically detrimental disease in dairy cattle, causing significant reductions in milk production, milk quality, and animal welfare (Gonçalves et al., 2018). Two primary forms of mastitis exist: clinical mastitis (CM) and subclinical mastitis (SCM). Clinical mastitis is characterized by visible changes in milk quality, udder inflammation, and systemic signs, whereas subclinical mastitis lacks observable clinical symptoms but is associated with decreased milk quality and elevated somatic cell counts. Subclinical mastitis and clinical mastitis have different epidemiological patterns and underlying causes. SCM is generally more prevalent than CM (subclinical mastitis accounts for approximately 90% of cases) and is often caused by suboptimal milking procedures, environmental factors, and contagious pathogens, such as Staphylococcus aureus and Streptococcus agalactiae (Birhanu et al., 2017). CM, on the other hand, is typically caused by more aggressive pathogens, including Escherichia coli and Klebsiella spp. Understanding these differences is crucial for implementing targeted preventive measures. The most apparent distinction between SCM and CM is the presence of clinical signs. Clinical mastitis presents with visible changes in milk, such as clots, flakes, or discoloration, as well as swelling, heat, and pain in the udder (Cheng&Han, 2020). In contrast, SCM lacks observable clinical signs but is associated with elevated somatic cell counts.

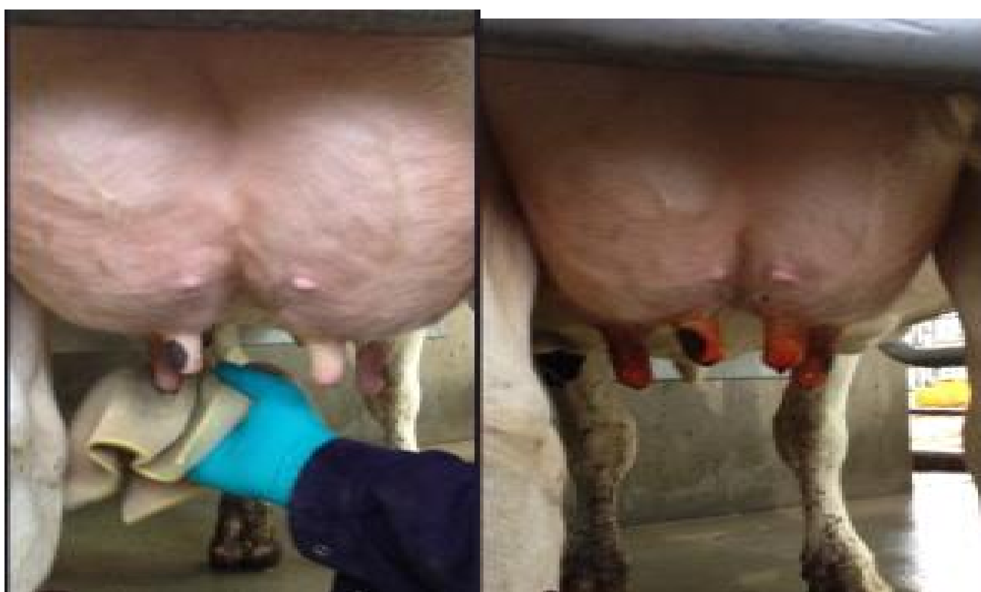


Figure 7. Differences between healthy udder and infected by mastitis.

Diagnosis of SCM is primarily based on somatic cell count analysis, while CM is diagnosed through clinical examination, microbial culture, and somatic cell counts. Both subclinical and clinical mastitis results in economic losses for dairy producers, but the extent of the impact differs. Subclinical mastitis leads to reduced milk production, poor milk quality, and increased veterinary costs due to interventions. Clinical mastitis incurs additional costs related to veterinary treatment, discarded milk, management labor, and decreased future milk production potential of affected cows. Accurate estimation of economic losses aids in decision-making for mastitis prevention and control strategies. Efficient management strategies vary between SCM and CM due to their unique characteristics. Preventive measures for SCM focus on maintaining proper milking hygiene, minimizing exposure to environmental pathogens, and implementing regular udder health monitoring. Management of CM involves prompt detection and treatment, as well as implementation of hygiene practices to prevent pathogen transmission within the herd. The selection of appropriate antimicrobial treatments and adherence to withdrawal periods are crucial to prevent antibiotic residues in milk.

In conclusion, subclinical and clinical mastitis are distinct forms of udder inflammation with varying epidemiological, clinical, economic, and management characteristics. Comprehensive understanding of these differences is vital for effective mastitis prevention, early detection, and management strategies (Mammadova&Keskin, 2013). By addressing both subclinical and clinical mastitis, dairy producers can work towards improving milk production, milk quality, and overall herd health, resulting in sustainable and profitable dairy operations.

1.4.2. EPIDEMIOLOGY OF MASTITIS

Mastitis can be caused by different types of microorganisms which can be classified as either environmental or contagious.

Environmental bacteria, as the name says, are derived from the cow's environment (bedding, soil manure, etc.) and are thus strongly affected by management measures. As they are endemic to the area where the animals stay, they cannot be eliminated and can only be controlled by enhancing the

cleanliness of both the cows and their environment. In short, the primary source is the infected udder, and the primary source of pathogens causing environmental mastitis is a contaminated environment. The most prevalent environmental bacteria are the coliforms (*E. coli, Klebsiella spp*, and *Enterobacter*), which originate in manure and soil, and the environmental streps (*S. uberis* and *S. dysgalactiae*) that come from the environment but also from infected udders. So environmental streps are present in the udder, it can also enhance the likelihood of them being contagious (Garcia, 2004).

Contagious bacteria are transmitted from an infected udder to a healthy cow and can spread from infected quarters to others. Pathogenic bacteria are typically transferred between cows during milking. Hands, towels, and the milking machine can be reservoirs for contagious bacteria.
The main contagious pathogens and environmental pathogens are respectively, *Streptococcus agalactiae*, and *Staphylococcus aureus subsp. aureus*, *Mycoplasma spp.*, environmental streptococci (streptococci other than *S. agalactiae* such as *Streptococcus uberis*; enterococci), *Enterobacteriaceae*, and coagulase-negative staphylococci. More than 140 different microorganisms have been identified from bovine intra-mammary infection, but the generality of bovine mastitis is mostly caused by Streptococcus species, like *S. agalactiae*, *S. dysagalactiae*, the leading organism is *Staphylococcus aureus*. It's usually seen in two forms, clinical &subclinical mastitis. *Escherichia coli* and *S. uberis* are associated with clinical mastitis, while *S. agalactiae* and *Enterococcus spp*. are associated with subclinical mastitis. Moreover, *S. aureus* is the causative pathogen of both clinical& subclinical mastitis (Cobirka et al., 2020), (S.A.R. Institute, 2016).

1.4.3. RISK FACTORS OF MASTITIS

Various risk factors related to the incidence of bovine mastitis play a considerable role, including pathogen, host, and environmental factors. All these factors were taken into importance in the mastitis examinations.

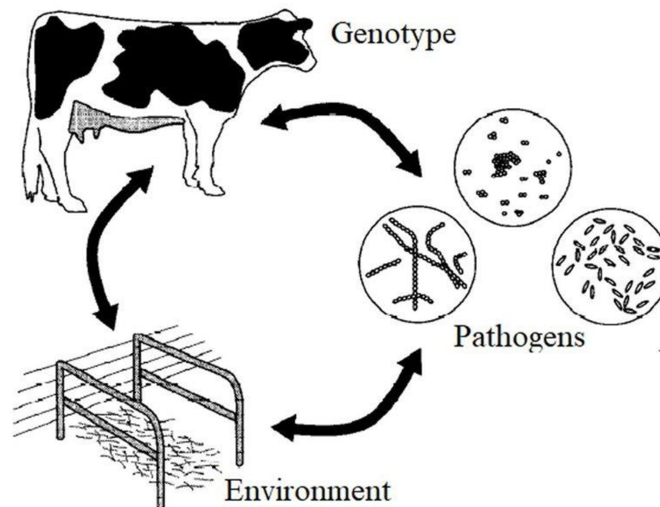


Figure 8. Pathogenicity of mastitis (A. Rasheed, 2020).

Pathogen factor

Intra-mammary infection (IMI) has been described as the primary reason for bovine mastitis, and many types of bacteria have been recognized as causative agents of bovine mastitis. Infectious pathogens are mostly transmitted from cow to cow during milking and live in the cow's udder or teat skin, colonize, and develop into the udder canal. Consequently, it may increase SCC (somatic cell count) and subclinical infection. Contagious pathogens can control the contact between healthy cows from infected cows with practices such as sterilization of the transmission or disinfection of the udder. Environmental pathogens do not live in the udder, but can be found in the bedding, and herd's house. Environmental pathogens can be controlled by reducing exposure to udder from environmental pathogens or antibiotic treatment (Cheng&Han W. C., 2020).

Host Factor

There are too many factors that are related to host factors, these are breeding and genetics, udder structure, age, transition period, host nutritional stress, and immune system. Genetic factor has shown that it has effects on mastitis in studies conducted to date. For example, high-yielding pure-breed cattle were found to be genetically more vulnerable to mastitis, especially compared to Holstein-Friesian cattle (Washburn, 2002). The study by S.P. Washburn et al. reported that Jersey cattle were

reported to have a lower rate of mastitis than Holstein-Friesian cattle. The udder structure of cows and the age of cows are associated with infection. The shape and size of the cow's udder and the teat to floor distance, especially cattle with large funnel-shaped teats or pendular-shaped udders are at risk for subclinical mastitis (Visscher et al., 2016). In addition, older cows are more sensitive to infection and the risk of bacterial contamination is higher because their udder canals are wider. N. Sharma et al. (2011) reported a high incidence of mastitis because of immunosuppression, related to high levels of oxidative stress and decreased antioxidant defense during the transition period which is the period between three weeks before and after birth (Sharma et al., 2011).

Environmental Factor

Environmental conditions practices such as keeping the environment clean significantly affect animal health and welfare and reduce the incidence and severity of mastitis. Many things need to be considered here, these are high stocking density, contaminated floors, wet bedding, poor ventilation, and a hot and humid climate.

1.4.4. SOMATIC CELL COUNT (SCC)& DIFFERENTIAL SOMATIC CELL COUNT (DSCC)

The most used parameter for a rapid screening of animals is the somatic cell count (SCC) in milk by Fossomatic test, an automatic cell counter widely used as it can analyze many samples per hour (Blowey&Edmondson, 2010). The threshold value for the commercialization of bulk milk is indicated by Reg.CE 853/2004, with 400,000 cells per mL (cell/mL). At the single quarter level, however, it is generally lower than 200,000 cells/mL (in healthy pluriparous cows) and for primiparous cows, it drops further up to 100,000 cells/mL (McCubin et al., 2022). These are considered good indicators of the animal's state of health (Sharma et al. S. N., 2011). Somatic cells are mainly composed of leukocytes, in particular lymphocytes, macrophages, and polymorphonuclear cells (PMN), and to a small extent cells deriving from desquamation of the mammary epithelium. These cells of the immune

system play a very important role in what is the inflammatory response within the mammary system. Lymphocytes are regulators of the induction and suppression of the immune response. Macrophages, which can actively phagocytose components "foreign" to the cell, initiate the immune response by recognizing invading pathogens, recruiting, for example, PMNs, whose role is to defend against bacteria during the first part of the acute inflammatory process (Holm et al., 2017). Exceeding these levels does not always indicate mastitis. The number of SCs that can be found during the analysis also varies according to factors such as age, stage of lactation, stress, season, and the moment in which the count is performed (Harmon, 1994). Another parameter to take into consideration when carrying out the count is the distribution of leukocytes, which changes depending on whether the milk was produced by an animal in a physiological state or by one in a pathological state. Specifically, in mastitic milk, PMNs can also reach percentages of about 95%, while in normal milk, mainly macrophages and lymphocytes will be found (as well as a low number of SCs) (Holm et al., 2017). Therefore, although the SCC is a valid parameter for the identification of the mammary gland inflammatory state, it is not able to give information on the distribution of PMN, lymphocyte, and macrophage populations (Stocco et al., 2020). The most recent studies led to the need to develop a new parameter, the differential somatic cell count (DSCC), which, if used in combination with SCC, could help at better evaluate the mammary gland status. DSCC represents the combination of PMN, and lymphocytes, expressed as a percentage. The proportion of macrophages can be calculated as a complement to 100 of the DSCCs. Some methods, such as microscopy or flow cytometry, although they allow for accurately discriminate of the different leukocyte populations (unlike DSCC which returns a cumulative proportion of PMNs and lymphocytes), cannot be considered for a routine analysis due to their performance characteristics (low repeatability and large time expenditure for microscopy, high cost and low accuracy for flow cytometry). The tool that therefore allows the high-yield screening of milk for SCC and DSCC is the Fossomatic™, whose accuracy is low when the number of DSCC drops below 50,000 cells/mL (Zecconi et al., 2020). This aspect should not be underestimated since, as described by Zecconi et al. (2020), for example the number of cows with

these values in a region such as Lombardy (the largest producer of milk in Italy) the figure reaches 20%. This could therefore lead to a loss of interest in the routine measurement of DSCC in milk samples, especially if one considers that even at values lower than 50,000 cells/mL cases of intramammary infections developing in the single quarter were still found. Despite this, the authors of the cited article have seen that even at values lower than 50,000 cells/mL, the increase in DSCC is correlated to a change in the composition of the milk, and therefore the parameter could be used as a marker of an alteration of milk secretory mechanisms. Currently, the developed method can be used to improve disease screening during Dairy Herd Improvement (DHI) programs, as, together with the already well-characterized SCC, it provides more accurate information on udder health status and specific stages of the pathology (e.g., clinical vs. sub-clinical infection) for values higher than the accuracy threshold of the instrument. In detail, thanks to data on the distribution of the various cell populations in milk, it is possible to detect the early onset of intramammary infections (very high percentage of PMN with somatic cell values below 400,000 cells/mL), the presence of chronic mastitis (high percentage of macrophages in counts greater than 400,000 cells/mL) or good udder health (low counts and high proportion of macrophages) (Holm et al., 2017).

As seen previously, the combination of the two indicators (SCC and DSCC) can be effectively used in mastitis screening procedures. These two parameters can also be associated with characteristics such as milk yield and composition. In a recent study proposed by Bobbo et al. (2020), it has been seen that depending on the state of health of the cows (healthy, susceptible, mastitis, or chronic), these characteristics undergo variations. More specifically, susceptible animals produce milk with 20 lower percentages of fat and protein, as a possible result of proteolytic and lipolytic activity stimulated by neutrophil recruitment. In chronically infected cows, on the other hand, these percentages increase, as the yield in terms of kilos of milk produced per day decreases significantly, thus leading to a concentration of the various nutritional components. As regards animals in which mastitis is in progress, it is possible to observe, as previously demonstrated in other studies, a reduction in the percentage of lactose, an event which can be explained both by the damage caused by the disease to

the mammary tissue with consequent impairment of synthesis, and by the increase in membrane permeability and the passage of lactose in the blood.

1.4.5. CONSEQUENCE OF MASTITIS

Mastitis leads to several negative aspects related to animal health and human health, the quality and production of milk, and economic consequences for the farmer. There is a danger that the bacterial transmission of milk from affected cows may present it unsuitable for human consumption by causing food poisoning and ensuring a mechanism of spread of disease to humans through the consumption of raw milk (Dagnaw, 2015). From an economic perspective, several aspects should be taken into consideration. The decrease in milk production by cows affected by clinical or subclinical forms of mastitis is recognized as the main cause of losses related to the disease (considerable as unreceived revenues) alongside a reduction in bonuses related to milk quality (content of fats, proteins, somatic cells, and bacterial load). To these parameters must be added all those parts of the costs related to health treatments and preventive measures that are applied in the event of the presence of mastitic cows in the herd. In the short term, the lethality of the pathology for animals must be considered but in the long term, the lowering of the animal's longevity must be considered. Among its consequences, mastitis also decreases the chances of maintaining and carrying the pregnancy to term. Antibiotics for treating and controlling mastitis may have possible implications for human health, as they increase the risk of the emergence of antibiotic-resistant bacterial strains and their entry into the food chain. Furthermore, when the withdrawal times before slaughtering the treated animals are not respected, antibiotic residues can also be found in the meat (Bradley, 2002). Another potential risk is the spread of zoonoses, especially in those situations where raw milk is consumed, but also through the purchase of products based on raw milk. Many farm families simply consume raw milk because it is a traditional practice, and it is cheaper to take milk from the bulk tank than to buy pasteurized retail milk. Some believe that raw milk has a higher nutritional value than pasteurized milk (Dagnaw, 2015). Reduction in milk production is an obvious symptom of clinical mastitis. The reduction in

production resulting from subclinical mastitis depends on the degree of inflammation, which can be estimated from the count of somatic cells in the milk. As the number of somatic cells increases, increasing reductions in milk production are found at both the individual and herd levels. Decreases in milk production at the individual and herd levels are associated with increased somatic cells. As the degree of inflammation increases, the milk chemical composition approaches that of the blood more and more because of the alteration of the permeability of the membranes at the mammary level, which facilitates the filtration of blood components from the bloodstream to the udder, and the reduction of the synthesis activity by the secretory tissue. The production of total solids, fat, casein, and lactose is substantially reduced while the total amount of protein changes only slightly due to the concomitant increase in immunoglobulins. The reduction of these components harms the technological characteristics of the milk, as well as a reduction of its shelf-life. The usage of genetic selection for the strength of mastitis can lead to a permanent improvement which translates into less dependence on antibiotic treatments, useful for preventing the formation of new antibiotic-resistant bacterial strains, a problem that is now widespread in the zootechnical environment in general (Shook, 2018).

2. AIM OF THE THESIS

This study was conducted within the LATSAN project, which aimed at developing innovative tools for improving mammary gland health and milk quality in dairy cattle.

The current thesis work focused on the evaluation of the existing relationship between subclinical mastitis and mammary gland inflammation status, and the detailed milk protein profile (including some minor components such as lactoferrin) in 450 lactating Holstein cows with the usage of RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography). Moreover, for the first time, we also evaluated the effect of the interaction between the intramammary infection and inflammation status on the protein fractions, to better evaluate the extent of alteration of the protein profile. The experimental unit was the mammary quarter within cow; therefore, all comparisons were made between positive and negative quarter samples as well as between different classes of SCC, within cow. Recognizing this scholarly gap and to provide a more complete level of information on this topic, the primary objective of this study was to probe the associations between naturally occurring subclinical IMI, four distinct pathogens (namely, *Staphylococcus aureus, Streptococcus agalactiae, Streptococcus uberis, and Prototheca spp.),* in combination with SCC on the detailed milk protein profile assessed at the quarter level in Holstein cows. The purview of this research extended to the quarter-level examination within Holstein cows, offering a refined perspective.

3. MATERIALS & METHODS

3.1. EXPERIMENTAL DESIGN AND SAMPLING

Milk samples were collected from 450 lactating Holstein cows kept on three commercial dairy farms in the Veneto region (northern Italy; 144, 71, and 235 in herds A, B, and C, respectively) for the study. The herds were chosen based on a preliminary survey conducted in the Veneto region by the State Veterinary Laboratory for Northeastern Italy (IZSVe) to determine the herd prevalence of *Streptococcus agalactiae, Staphylococcus aureus, Streptococcus uberis*, and *Prototheca spp.* Cows were housed in fully accessible stalls and fed a TMR diet of corn silage, sorghum silage, and concentrates. Milking took place twice a day, and drinking water was provided in automatic water bowls. Milking was done twice a day, and drinking water was available in automatic water bowls. Farmers and their local veterinarians were in full control of animal health, observing animals and performing exams as needed.

Sampling was carried out at three different times as reported in Figure 9.

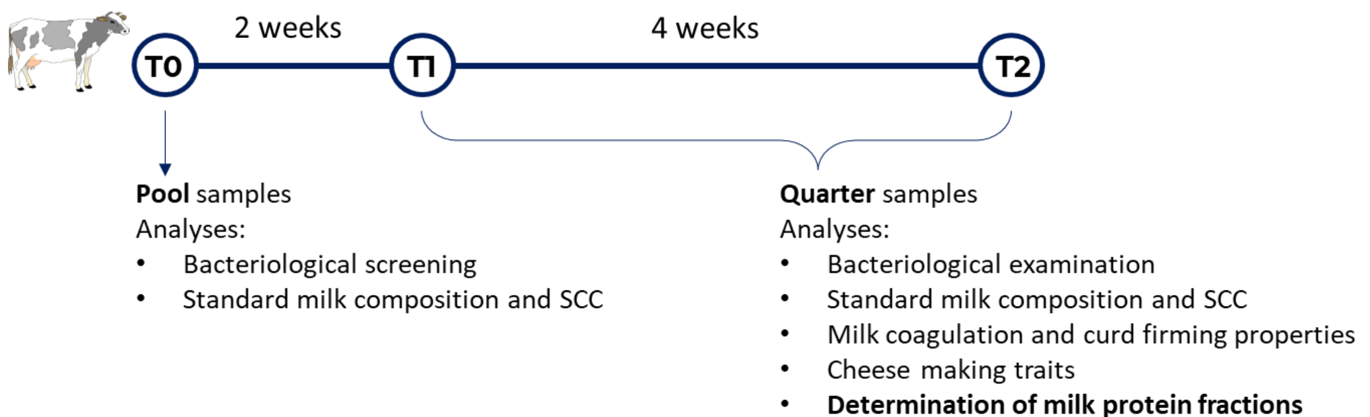


Figure 9. Schematic representation of the experimental design and sampling activity. T0: Time 0, T1: time 1, after two weeks from T0; T2: time 2, after four weeks from T1. SCC: somatic cell count. Modified from Pegolo et al., (2022).

The first sampling of composite milk carried out at time 0 (T0), allowed us to perform a bacteriological screening and to identify negative and positive animals. From the latter were excluded all the animals with clinical signs and animals with previous history of antibiotic and drug treatments.

A total of 78 animals were selected and subjected to two subsequent single-quarter milk samplings (T1, after two weeks, and T2, after six weeks) to monitor the progression of the disease. Samples for bacteriological screening were performed by manual sterile milking which was carried out first by cleaning externally the teat ends with commercial pre- milking disinfectants, drying them with individual towels, and finally cleaning them again with alcohol. After removing the first flows of foremilk, up to 10 mL of milk from each quarter was obtained in sterile tubes and pooled at T0, collected in different tubes at T1 and T2. Milk samples were kept on ice until they were refrigerated at 4°C and transferred to the laboratory of Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe). Immediately following the sterile collection of milk samples for bacteriological analysis, approximately 150 mL of milk was manually collected in one container from all cows (T0) or all quarters (T1 and T2), divided into three subsamples, and kept at 4°C.

Finally, one subsample (50 mL) with bronopol was delivered to the laboratory of the Veneto Regional Breeders Association (ARAV) for the milk composition analysis and SCC determination, and two subsamples (50 mL) without preservatives were transported to the milk laboratory of the Department of Agronomy, Animals, Food, Natural Resources, and the Environment of the University of Padova. Subsamples were obtained from the latter for the evaluation of the detailed protein profile of the milk; aliquots were stored at -80°C until the analyses were performed.

3.2. MICROBIOLOGICAL ANALYSIS, MILK COMPOSITION AND SCC DETERMINATION

Milk samples were frozen after delivery to the laboratory and tested in 3d for microbiological analysis at the IZSVe.

Selective media used in this analysis are as follows;

Baird Parker agar with rabbit plasma fibrinogen (BP-RPF, Biokar Diagnostic, Beauvais, France), prepared following the ISO 6888-2 (1999) standards, for the identification of *S. aureus*; thallium kristalviolette tossing agar (IZSVe internal production), according to the method described by Hauge

and Ellingsen (1953), for the identification of *S. agalactiae*; *Prototheca* isolation medium (PIM, IZSVe internal production), prepared in accordance with the NMC guidelines (NMC, 2017) (Hauge&Ellingsen, 1953).

Microbiological milk analysis followed the steps below:

- The milk composite samples were inoculated 10 µL from on selective media at 37°C.
- Growth on Baird-Parker agar base plates was observed after 24 and 48 hours of incubation. A tube coagulase test was used to confirm suspicion of *Staph. aureus* colonies.
- At 24 hours, Thallium sulfate-crystal violet-B toxin blood agar plates were observed, as were suspected *Strep* colonies. The Christie-Atkins-Munch-Peterson test was used to confirm the presence of *agalactiae*.
- At 24, 48, and 72 hours, Prototheca isolation medium plates were observed, and suspected colonies were confirmed using the wet mount method.
- Quarter-milk samples were cultured following NMC recommendations.
- Bacterial colonies with questionable or unclear test results were identified using MALDI-TOF MS on a Microflex Biotyper LT (Bruker Daltonics GmbH). The log (score) 2.0 was used as the species-level identification threshold.

Milk composition analysis was carried out at the laboratory of the Veneto Regional Breeders Association (ARAV) using the Milkoscan infrared Analyser (Foss A/S, Hillerød, Denmark) while SCC was determined using the FossomaticTM 7 DC analyzer 155 (Foss A/S, Hillerød, Denmark).

3.3. DETAILED MILK PROTEIN FRACTION DETERMINATION

3.3.1. REAGENTS, CHEMICALS& STANDARDS

3.3.1.1. SAMPLE PREPARATION

For the separation of the emulsion phase from the other two phases, two solutions based on Guanidine hydrochloride (GdnHCl) are used, with different molarities.

For 50 mL GndnHCl 6M, the following were used:

- 28.66 g of GdnHCl (Sigma-Aldrich) (lot G-4505, purity >99%);
- 1.05 g of Bis-tris Buffer (Sigma-Aldrich) (lot B-9754, >98%);
- 0.057 g of sodium citrate (Sigma-Aldrich) (lot 71498, >99%);
- 0.15 g of Dithiothreitol (DTT) (Sigma-Aldrich) (lot 43817, >99%).

All dissolved in 50 mL of ultra-pure water (Milli-Q gradient, Q-Gard® 1, Merck Millipore, Darmstadt, Germany)

For the GndnHCl 4.5 M solution, the following are used:

- 21.49 g of GdnHCl dissolved in solution A.

3.3.1.2. Phase preparation for HPLC

Two solutions, solution A and solution B, were used for HPLC, the following were used.

Solution A

- 94.9% ultra-pure water (Milli-Q System, >18.2 MΩ cm);
- 5% acetonitrile (ACN) (Sigma-Aldrich, St. Louis, MO, USA);
- 0.1% trifluoroacetic acid (TFA) (Sigma-Aldrich, St. Louis, MO, USA); 26 (lot T-6508,> 99%).

Solution B

- 99.9% ACN (Sigma-Aldrich, St. Louis, MO, USA);

- 0.1% TFA (Sigma-Aldrich, St. Louis, MO, USA);

3.3.1.3. CALIBRATION CURVES

Curves were produced for the quantification of the various protein fractions in the samples calibration using standard protein solutions purchased from Sigma (Sigma-Aldrich, St. Louis, MO, USA):

- κ-CN (lot C-0406, >80%)
- α-CN (lot C-6780, >70%)
- β-CN (lot C-6905, >90%)
- α-Lactalbumin (lot L-5385 type I, ~85%)
- β-Lactoglobulin, variant B (lot L-8005, >90%)
- β-Lactoglobulin, variant A (lot L-7880,>90%)
- Lactoferrin (lot L-9507, >85%)

3.4. RP-HPLC (REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY)

Chromatography is a method for separating components in a mixture, using two phases, stationary and mobile and it separates the components according to their proximity to these phases.

In RP-HPLC, the stationary phase should be non-polar and it can be mounted on planar support or a column and can be found in a solid or liquid state (in this case adsorbed on a solid port); the mobile phase can be found in a liquid or gaseous state and must meet requirements such as the ability to solubilize the sample, the absence of corrosivity and toxicity, the absence of interaction with the fixed phase, and a high degree of purity (acetonitrile and methanol are generally used). RP-HPLC is focused on hydrophobic interactions and can measure these interactive forces based on the reversible adsorption/desorption of hydrophobic solute molecules, reversed-phase separation experiments are

carried out in several basic steps, as shown in Figure 10. To facilitate interactions with proteins, 0.1% trifluoroacetic acid is added to these solvents composed of water, acetonitrile, or methanol. The reverse phase separation mechanism depends on the hydrophobic interactions between the solubilized molecules in the mobile and stationary phases. The initial conditions in which the mobile phase is found are aqueous. This leads to a structuring of the water molecules around the unbound solutes. When they bind to the hydrophobic ligand immobilized in the fixed phase, the degree of organization of the water molecules decreases, consequently the entropy of the system increases, and the association between solute and ligand is favored.

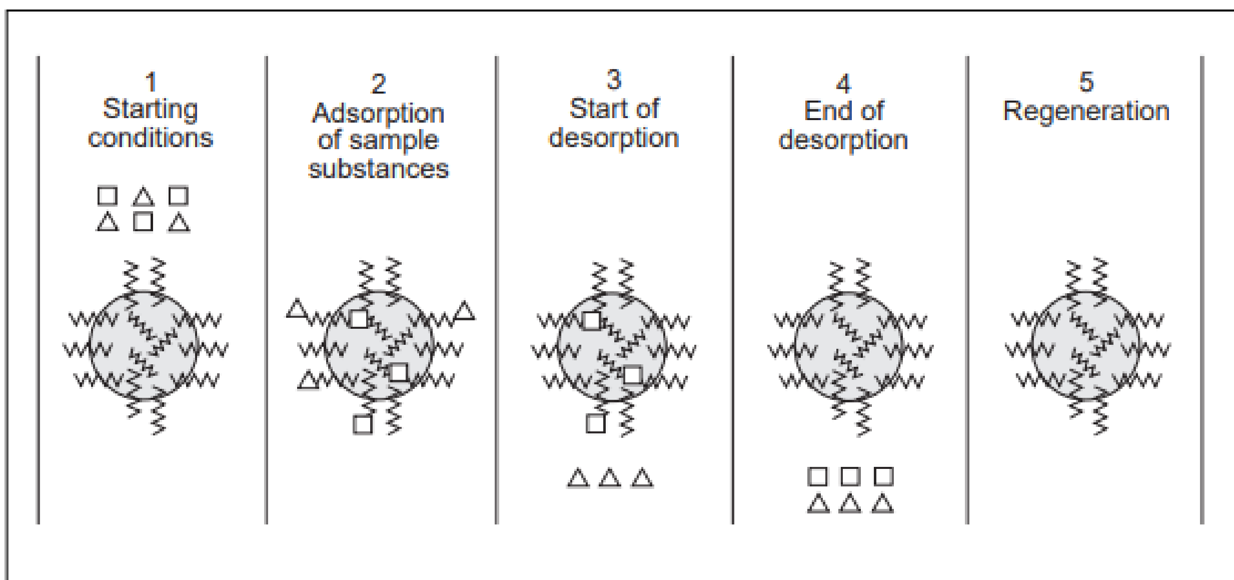


Figure 10. Principle of Reverse Phase Chromatography with Gradient Elution(Biosciences, 1999).

RP-HPLC steps are generally composed of:

- The first step of the chromatograph is to stabilize the column with the reverse phase under (ionic strength, and polarity conditions) the preliminary mobile phase is appropriate for the mobile phase, and for controlling the mobile phase polarity, organic modifiers are used. The initial mobile phase polarity (known as mobile phase A) must be low enough to dissolve the

partially hydrophobic solute while remaining high enough to ensure solute binding to the reversed-phase chromatographic matrix.

- The second step, the sample containing the solutes to be separated is applied. The sample should ideally be dissolved in the same mobile phase that was used to equilibrate the chromatographic bed. The sample is applied to the column at a flow rate that will result in the best binding. Following the application of the sample, the chromatographic bed is washed with mobile phase A to remove any unbound and unwanted solute molecules. Bound solutes are then desorbed from the reversed-phase medium by adjusting the mobile phase polarity, causing the bound solute molecules to desorb and elute sequentially from the column. This usually entails increasing the percentage of organic modifiers in the mobile phase to decrease the mobile phase polarity. This is accomplished by keeping the final mobile phase at a high number of organic modifiers. The initial and final mobile phase pH solutions are usually the same. Mobile phase A contains a small or no organic modifier, whereas mobile phase B contains a greater amount of organic modifier, which causes a gradual decrease in mobile phase polarity from A to B.

- The third step includes removing previously desorbed substances by switching mobile phase B to a nearly 100% organic modifier to ensure the complete removal of all bound substances before reusing the column.

- The fourth step is to return the chromatographic medium to the initial mobile phase conditions after it has been exposed to 100% mobile phase B. The different binding properties of the solutes present in the sample because of their hydrophobic properties cause separation in reversed-phase chromatography. The hydrophobic properties of the initial mobile phase can be used to control the degree of solute molecule binding to the reversed-phase medium.

Although the hydrophobicity of a solute molecule is difficult to quantify, the separation of solutes with only minor differences in hydrophobic properties is simple. Because of its greater resolving power, reversed-phase chromatography is an effective method for the high-performance separation of complex biomolecules (Biosciences, 1999).

As a result, the lipophilic substances, for example, solute and ligand, benefit from the association. The reverse phase chromatography technique offers great versatility and advantage in separation conditions because it allows the desired solvent to pass through the column while choosing whether to bind to contaminants. In general, researchers favor solvent binding. Because the desorbed solute elutes from the chromatographic medium in a concentrated form when the solvent is bound.

Also, since binding is complete under initial mobile phase conditions, the initial concentration of the desired solute in the sample solution is not critical and allows dilute samples to be used in the column. Reversed-Phase High-Performance Liquid Chromatography (RP-HPLC) has become a well-established biomolecule analysis and purification tool. The ability of RP-HPLC to separate polypeptides of almost identical sequences, not only for minor peptides such as those obtained through trypsin digestion but additionally for much larger proteins, is the reason for its main role in analyzing and purification of proteins and peptides.

3.5. SAMPLE PREPARATION FOR RP-HPLC (REVERSE PHASE-HIGH PERFORMANCE LIQUID CHROMATOGRAM) ANALYSIS

The preparation of milk samples was made following the procedures of a validated method reported in the work of Maurmayr et al. (2013), and consisted as follows:

The milk samples were stored at -80 in the freezer then the samples were thawed at room temperature (20-22 ºC). After thawing, 500 µl milk sample and 500 µl GdnHCl 6M was added to eppendorf tubes (Figure 11.1) and mixed with a vortex mixer for 10 seconds for the sample to be homogenized.

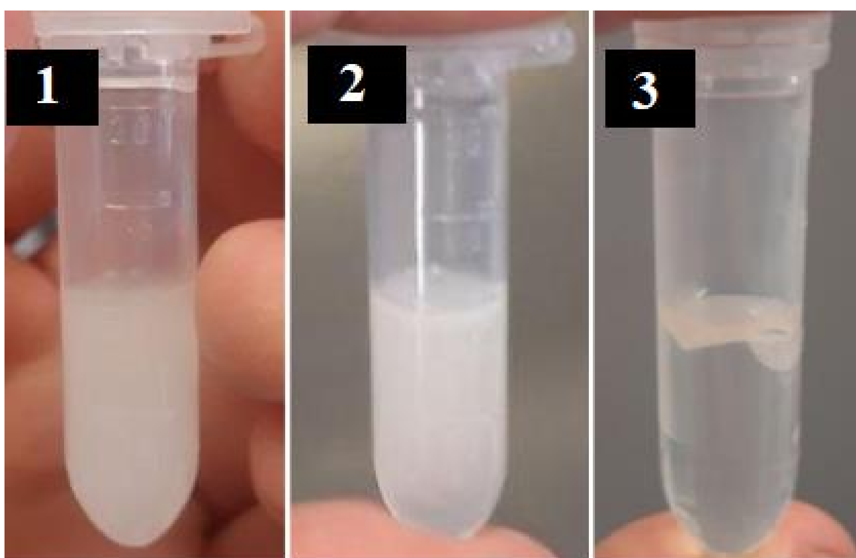


Figure 11. 1) Milk sample and GdnHCl 6M; 2) Milk sample after incubation; 3) Milk sample after centrifugation.

After mixing, the samples were left to incubate for one hour at room temperature to separate fat from milk (Figure 11.2). Then the milk samples were centrifuged at 13,000 RPM for 10 minutes to remove the fat. In the end, the lipid portion can be seen in a superficial layer (Figure 11.3). After centrifugation, a small amount is taken from the sample (250 µL) with great attention to not damaged the fat layer and placed in a new eppendorf containing 750 µL of 4.5 M GdnHCl solution. As the last step before transferring into 2 mL vials (Agilent Technologies) for the HPLC run, the samples are filtered through a 0.45 µm filter (GVS North America) using a 1 mL syringe (Omnifix®-F - B|Braun), to eliminate any impurities that may interfere with the chromatographic analysis. In addition to the milk samples, GND 6M is added to a vial to clean the columns after every four samples (Maurmayr, 2013).

3.6. CHROMATOGRAPHIC CONDITIONS

The instrument used is an Agilent 1260 Series chromatograph (Agilent Technologies, Santa Clara, CA, USA) controlled by the Agilent ChemStation for Lc System software, which allows the

acquisition, processing of data and setting of the various run parameters. For running the samples in HPLC, a preliminary conditioning phase of the column is required, which is conducted by slowly rising the instrument flow from a pressure of 0.1 mL/min to 0.5 mL/min. This conditioning phase is also important for allowing the rising of the temperature of the column to 70°C and for the column back pressure to stabilize at 330-340 Bar. At this point, the autosampler (Agilent 1100 series, G1313A) takes a volume from the vials of 2 µL which is injected into a C8 reversed-phase analytical column (Aeris WIDEPORE XB C8, Phenomenex) with a stationary phase of 3.6 µm, 300 Å, 250 x 2.1 I.D.

The following steps were taken to optimize analytical quality and time requirements:

- Gradient elution was performed using a mixture of solution A and solution B solvents.
- The following program was used to separate the bovine protein fraction: linear gradient from 20 to 29% B in 0.5 min, from 29 to 33% B in 5.5 min, from 33 to 36% B in 6 min, from 36 to 45% B in 6 min and return linearly to the starting condition in 1 min.;
- Before injecting the next sample, the column was re-equilibrated under starting conditions for 3 minutes, and the total analysis time per sample was 22 min.;
- the flow rate was 0.5 ml/min;
- the column temperature was kept at 70°C.

In the end, the eluate is read by the detector (Agilent Technologies 1260 Infinity, G1315C) at the wavelength of 214 nm. Through these separation conditions, it is possible to identify not only the different protein fractions but also the single genetic variants of κ-CN (A/B), β-CN (B/A1 /A2 /I-H 2), and β-LG (Maurmayr, 2013).

3.7. INTEGRATION AND QUANTIFICATION OF CHROMATOGRAMS

The Agilent Chemstation for Lc System software makes it possible to proceed with integrating and quantifying the chromatograms produced during the HPLC run. For this purpose, calibration lines have been created for each protein fraction using standards at different concentrations referred to the method of Maurmayr et al. (2013) (Figure 12).

Milk protein fraction:	Concentration mg/mL				
	A	B	C	D	E
κ-CN	1.05	1.40	1.87	2.50	3.33
α-CN[1]	2.10	2.81	3.75	5.00	6.67
Lactoferrin	1.68	2.25	3.00	4.00	5.33
β-CN	0.63	0.84	1.12	1.50	2.00
α-lactalbumin	0.42	0.56	0.75	1.00	1.33
β-Lg A or B	0.84	1.12	1.50	2.00	2.67

[1] for quantification it was applied a 4:1 proportion between α_{S1} and α_{S2} fractions

Figure 12. Concentration of the standard casein fractions (Maurmayr, 2013).

The 5-point calibration curves were used to quantify the sample by linear regression of the corresponding peak area and the concentration of injected standard. Individual quantified protein fractions were expressed in grams per liter (g/L) as well as a percentage of total milk nitrogen (%N).

3.8. STATISTICAL ANALYSIS

The association between quarter-level protein fractions and subclinical mastitis was explored using the SAS PROC MIXED procedure (SAS institute Inc., Cary, North Carolina, USA). Two separate analyses were conducted for T1 and T2 due to heterogeneity of the variances and to the possible change in bacteriological status of the quarter between T1 and T2. The following hierarchical mixed model, in which the experimental unit was the individual mammary gland quarter, was adopted:

$$y_{ijklmn} = \mu + \text{DIM}_i + \text{Parity}_j + \text{Herd}_k + \text{SCC}_l + \text{BACT}_m + (\text{SCC} \times \text{BACT})_{lm} + R_{n:ijk} + e_{ijklmn}$$

Where: y_{ijklmn} is the investigated trait (milk proteins); μ is the overall mean; DIM_i is the fixed effect of the *i*th class of days in milk (*i* = 3 classes; class 1 ≤ 120; 120 < class 2 ≤ 240; class 3 > 240); $Parity_j$ is the fixed effect of the *j*th parity (*j* = primiparous or multiparous); $Herd_k$ is the fixed effect of the *k*th herd/date (*k* = 3 herds); SCC_l is the fixed effect of the *l*th class of SCC (*l* = 4 classes; class 1 < 50,000 cell/mL; 50,000 cell/mL ≤ class 2 < 200,000 cell/mL; 200,000 cell/mL ≤ class 3 < 400,000 cell/mL; class 4 ≥ 400,000 cell/mL); $BACT_m$ is the fixed effect of the *m*th class of bacteriological status (*m* = positive or negative); $(\text{SCC} \times \text{BACT})_{lm}$ is the two-way interaction between SCC_l and $BACT_m$; $R_{n:ijk}$ is the random effect of the *n*th replicate/animal nested within DIM_i, $parity_j$, and $herd_k$; and e_{ijklmn} is the random residual. R and the residuals were assumed to be normally distributed with a mean of zero and variances of σ_r^2 and σ_e^2, respectively. With this nested design, the effects of DIM_i, $parity_j$, and $herd_k$ were tested on the error line of the *n*th animal nested within DIM_i, $paty_j$, and $herd_k$, hile the SCC_l, $BACT_m$, $(\text{SCC} \times \text{BACT})_{lm}$, and animal effects were tested on the residual term.

We also tested the position and number of affected quarters, but since no significant outcome was obtained, we excluded them for the final analyses.

For SCC classes the orthogonal contrasts were set as follows:

- class 1 + class 2 *vs* class 3 + class 4 (low SCC *vs* high SCC)
- class 1 *vs* class 2 (within low SCC)
- class 3 *vs* class 4 (within high SCC)

While for BACT x SCC interaction, orthogonal contrasts were built as following:

- negative (SCC class 1 + class 2 + class 3 +class 4) *vs* positive (SCC class 1 + class 2 + class 3 + class 4)
- negative (low SCC) *vs* positive (low SCC)
- negative (high SCC) *vs* positive (high SCC)

- negative *vs* positive (SCC class 1)
- negative *vs* positive (SCC class 2)
- negative *vs* positive (SCC class 3)
- negative *vs* positive (SCC class 4)

The significance was set at $P < 0.05$.

4. RESULTS AND DISCUSSION

This research assessed the impact of naturally happening subclinical intramammary infections (IMI), the inflammation condition indicated by somatic cell count (SCC) levels, and their interplay on milk protein components in quarter-level Holstein cows for the first time. This study was to evaluate the relationship between mastitis disease on milk composition and identify& quantify the protein fractions including some minor components such as lactoferrin in 450 lactating Holstein cows with the usage of RP-HPLC (Reversed-Phase High-Performance Liquid Chromatography).

4.1. Prevalence of Pathogens in Herds

The 450 cows sampled within the project were divided as follows within the 3 herds:

144 cows belonging to Farm 1, 71 cows in Farm 2 and 235 in Farm 3.

Table 1. Microbiological results of milk samples collected at time 1 (T1) and time 2 (T2).

Microbiological results	T1		T2	
	N	%	N	%
S. aureus	4	1.6	3	1.1
Strep. agalactiae	32	12.6	34	12.3
Prototheca spp.	13	5.1	8	2.9
Strep. uberis	3	1.2	1	0.4
Contaminated samples[1]	8	3.2	11	4.0

[1]Samples contaminated by environmental microorganisms. (*Acinetobacter* spp., *Bacillus* spp., *Corynebacterium* spp., *E. coli, Enterococcus* spp., *Klebsiella* spp., *Proteus* spp., *Staphylococcus* spp. *coagulase negative*)

Three different times, T0, T1 and T2, were used in the analysis. T0: time 0, pool samples; T1: time 1, 2 weeks after time 0, quarter samples; T2: time 2, 4 weeks after T1, quarter examples.

At T0, a total of 78 positive cows were identified and distributed among the pathogens as follows (Table 1);

- 51 positive animals for *Streptococcus agalactiae*;
- 19 positive animals for *Prototheca* spp.;
- 6 positive animals for *Staphylococcus aureus*;
- 5 positive animals for *Streptococcus uberis*;

The most prevalent pathogen was *Streptococcus agalactiae* at both T1(n=50) and T2(n=38).

According to Pegolo et al. (2022), they discovered no notable distinctions in milk yield between animals that tested negative and positive in the bacteriological analysis. This suggests that subclinical infections might not produce noticeable changes. In contrast, as anticipated, there was a significant dissimilarity in somatic cell counts (SCC) between animals that tested negative (199,700 cells/mL) and positive (688,600 cells/mL) ($P < 0.001$).

A total of 529 quarter milk samples were analyzed to identify and measure milk protein components. These samples were divided into two groups based on the time of collection: 253 were collected at T1, and 276 were collected at T2. Among these samples, 71 tested positive for microorganisms at T1, while the number decreased to 51 at T2. However, 19 samples (8 at T1 and 11 at T2) were contaminated, and therefore, they were not included in the statistical analysis. It's worth noting that *S. agalactiae* was the predominant pathogen, with 32 positive samples at T1 and 34 positive samples at T2.

4.2. Identification and Qualification of HPLC

A reverse-phase analytical column C8 (Aeris WIDEPORE XB-C8, Phenomenex, 3,6 m, 300, 250 x 2,1 I.D.) was used for the analysis. There are numerous techniques for the separation, identification, and quantification of single protein fractions based on various techniques such as electrophoresis, isoelectric focusing, mass spectrometry, and various types of HPLC (ion exchange, hydrophobic interaction, and reversed-phase) methods have been developed (Bonfatti et al., 2008). This is because it is important to examine the relationship between the protein profile and the technological properties of milk. The method created and approved by Maurmayr et al. (2013) was used in this work to separate protein fractions. This method involved a gradient run lasting a total of 22 minutes, with 19 minutes dedicated to complete sample elution and an additional 3 minutes for post-run column re-equilibration. The efficacy of differentiating between distinct protein fractions was assessed by comparing the HPLC data with the results illustrated in Figure 13. Notably, the data aligned well with the protein fraction profiles displayed in the figure, indicating successful protein fraction determination with substantial resolution.

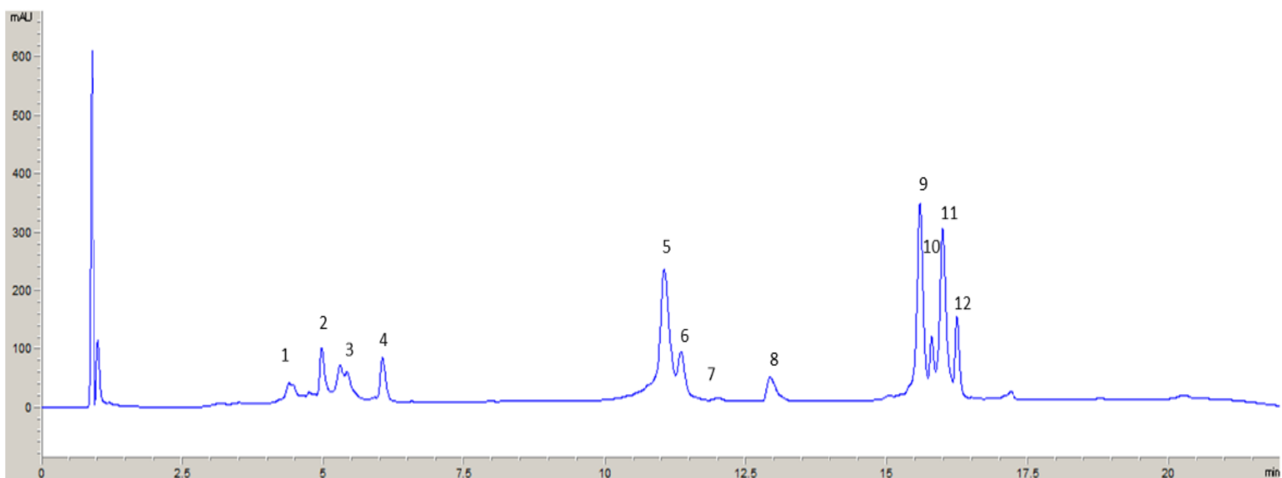


Figure 13. Chromatogram obtained from a single milk sample. n.1 = glycosylated kCN; n.2 = k-CN A; n.3 = αs2-CN; n.4 = k-CN B; n.5 = αs1-CN B; n.6 = αs1-CN C; n.7 = lactoferrin; n.8 = α-LA; n.9 = β-CN A1; n.10 =β LG B; n.11 = β-CN A2; n.12 = β-LG A.

Samples that were potentially affected by milk deterioration or contamination were excluded from the study. This decision was made due to the impracticable chromatogram results associated with these samples.

4.3. Descriptive Analyses

Table 2 provides a comprehensive overview of the descriptive statistics for milk protein fractions, presented both quantitatively (in grams per liter, g/L) and qualitatively (as a percentage of nitrogen, %N).

Table 2. Descriptive statistics of the milk protein fractions expressed both quantitatively (g/L) and qualitatively (%N).

Item[1]	N	Mean	SD	P1[2]	P99[2]
Protein composition, g/L of milk					
True protein	528	33.60	4.04	24.43	43.09
Caseins	528	27.92	3.28	19.98	35.07
κ-CN	528	5.49	1.40	3.27	9.58
Glycosylated κ-CN	528	2.13	0.86	1.03	5.33
Non glycosylated κ-CN	528	3.36	1.09	1.56	6.87
αs2-CN	528	3.36	0.67	1.98	5.07
αs1-CN	528	9.26	1.42	6.31	13.00
β-CN	527	9.83	2.19	5.30	14.50
Whey proteins	528	5.68	1.33	3.41	10.20
β-LG	528	4.47	1.19	2.32	8.30
α-LA	528	1.04	0.25	0.61	2.08
LF	526	0.16	0.19	0.03	0.97
N compound	499	2.45	1.02	0.28	4.81
Urea	524	0.22	0.05	0.12	0.36
Minor N compound	483	1.87	0.96	0.11	4.23
Protein composition, % of total milk N					
True Protein	503	93.20	2.65	87.51	99.17
Caseins	528	77.81	1.13	74.05	79.73
κ-CN	528	15.24	3.07	10.18	24.78
Glycosylated κ-CN	528	5.87	1.98	2.95	12.65
Non glycosylated κ-CN	528	9.37	2.73	4.23	17.82
αs2-CN	528	9.36	1.53	5.63	13.50
αs1-CN	528	25.89	3.27	18.92	32.93
β-CN	527	27.35	5.17	16.51	36.91
Whey proteins	528	15.84	3.34	10.40	27.61

β-LG	527	12.47	3.06	7.01	21.18
α-LA	528	2.91	0.67	1.74	5.04
LF	526	0.44	0.49	0.10	2.52
N compound	499	6.79	2.65	0.82	12.49
Urea	483	1.55	0.63	0.47	3.12
Minor N compound	483	5.19	2.53	0.34	11.05

[1] κ-CN: sum of glycosylated κ-CN and carbohydrate-free κ-CN; Caseins= sum of κ-CN + α_{s2}-CN + α_{s1}-CN + β-CN; Whey proteins= sum of β-LG + α-LA + LF; NPN compounds= sum of Urea and Minor NPN compounds.
[2] P1 = 1st percentile; P99 = 99th percentile

The quantitative and qualitative traits of different milk protein fractions are detailed in Table 2. The mean, median, and standard deviation help us understand the variability and central tendency within each fraction. An alternative viewpoint on the protein composition is provided by the matching figures represented as a percentage of nitrogen (%N). These data can be used to identify patterns and fluctuations in the milk protein fractions, which will help us understand the composition better. -CN and S1-CN, the two casein fractions of relevance, showed average amounts of 9.83 g/L (27.35%N) and 9.26 g/L (25.89%N), respectively. With an abundance of 4.47 g/L (12.47%N), -LG was found to be the most common whey protein, followed by -LA (1.04 g/L, 2.91%N), and LF (0.16 g/L, 0.44%N). These recorded values were largely consistent with research results from earlier studies using composite milk samples and the same RP-HPLC methodology (Amalfitano et al., 2020; Bisutti et al., 2022). The validity of the study performed at the individual quarter level is highlighted by this congruence. The protein fraction patterns at the two sampling sites showed a general similarity.

4.4. Associations between subclinical intramammary infection and milk protein fractions

The outcomes of the ANOVA about to the impact of intramammary infections (IMI) on the studied traits have been carefully documented in Table 3.

Table 3. F-values and significances of the linear mixed model tested for quarter-level protein fractions at sampling time 1 (T1) and time 2 (T2).

Item[1]	T1 BACT[2]	T1 SCC[3]	T1 SCC*BACT[4]	T2 BACT[2]	T2 SCC[3]	T2 SCC*BACT[4]
Protein composition, g/L of milk						
True Protein	0.66	0.73	0.33	1.39	0.48	0.24
Caseins	0.00	1.91	2.16	0.61	0.97	0.67
κ-CN	5.94^{*}	18.67^{***}	1.00	0.19	8.86^{***}	0.27
Glycosylated κ-CN	2.24	3.52^{*}	1.51	0.01	9.99^{***}	0.33
Carbohydrate-free κ-CN	3.32	15.56^{***}	2.85^{*}	0.97	5.06^{**}	0.23
αs2-CN	9.15^{**}	3.96^{**}	0.48	1.20	6.41^{***}	1.19
αs1-CN	0.77	5.35^{**}	0.22	0.55	3.13^{*}	3.53^{*}
β-CN	6.97^{**}	4.05^{**}	1.33	0.34	3.53^{*}	0.31
Whey proteins	3.58	1.94	0.73	0.03	0.12	0.63
β-LG	2.92	2.69^{*}	1.03	0.44	0.20	1.03
α-LA	1.49	1.97	0.30	0.35	0.51	2.29
LF	1.72	19.24^{***}	2.11	3.92^{*}	5.77^{**}	1.58
N compound	1.56	2.03	1.09	0.02	5.83^{***}	0.63
Urea	0.96	10.98^{***}	0.35	3.22	8.26^{***}	0.44
Minor N compound	2.23	1.76	0.64	0.20	8.09^{***}	0.46
Protein composition, % of total milk N						
True protein	1.54	1.86	0.93	0.27	5.24^{**}	0.95
Caseins	4.81^{*}	27.64^{***}	4.58^{**}	0.13	27.57^{***}	0.72
κ-CN	5.95^{*}	12.75^{***}	0.87	0.03	8.68^{***}	0.79
Glycosylated κ-CN	1.68	2.83^{*}	1.84	0.03	9.13^{***}	0.53
Carbohydrate-free κ-CN	3.59	11.04^{***}	2.20	0.67	4.25^{**}	0.62
αs2-CN	9.47^{**}	2.35	0.43	0.72	2.78^{*}	0.79
αs1-CN	0.11	9.76^{***}	0.12	1.93	15.28^{***}	3.74^{*}
β-CN	10.36^{**}	5.19^{**}	1.26	0.02	9.51^{***}	0.24
Whey proteins	1.54	1.87	1.22	0.18	0.49	0.59
β-LG	1.75	3.15^{*}	1.64	0.70	0.67	0.81
α-LA	0.61	1.49	0.33	0.49	0.51	2.37
LF	0.55	16.90^{***}	1.19	4.38^{*}	5.91^{***}	1.83
N compound	1.54	1.86	0.93	0.24	5.11^{**}	0.92
Urea	1.18	18.21^{***}	1.10	1.40	5.98^{***}	0.47
Minor N compound	2.23	1.69	0.54	0.06	7.34^{***}	0.55

[1] κ-CN: sum of glycosylated κ-CN and carbohydrate-free κ-CN; Caseins= sum of κ-CN + α_{s2}-CN + α_{s1}-CN + β-CN; Whey proteins= sum of β-LG + α-LA + LF; NPN compounds= sum of Urea and Minor NPN compounds.

[2] BACT: Bacteriological status (negative, positive).

[3] SCC: Somatic cell count, expressed in classes (4 classes: Class 1: SCC $<$ 50,000 cells/mL, 50,000 cells/mL $\leq$ class 2 $<$ 200,00 cells/mL; 200,00 cells/mL$\leq$ class 3 $<$ 400,000 cells/mL; class 4 $\geq$ 400,00 cells/mL.

[4] BACT x SCC: Interaction between the bacteriological status and the somatic cell count effects.

Unfortunately, severe fragmentation of the information made it impossible to determine the precise impacts of individual infections on the examined features. Under these conditions, considering this pathogen's unique impacts would have significantly decreased the test's power. Small subgroup sizes for each infection were caused by the dataset's diversity, which included different pathogens present at different frequencies. This reduced the statistical power needed for meaningful analysis. The goal to retain statistical robustness and the capacity to reach meaningful results led to the choice to forego pursuing pathogen-specific effects. According to our data at T1, there is a clear correlation between the majority of casein fractions and subclinical intramammary infections (IMI).

Our findings specifically showed that the milk composition of mammary quarters that tested positive for bacteria after bacteriological examination had changed noticeably. Especially remarkable were the enhanced proportions of κ-CN (P 0.05, representing an increase of +5.8%) and s2-CN (P 0.01 reflecting an increase of +5.7%) in these quarters. The concentration of ß-CN, however, showed a substantial decrease (P 0.01, -7.5%) (Figure 14). The activity of immune cells like polymorphonuclear (PMN) cells and macrophages, which release many proteases and other cellular components in the milk while fighting invading pathogens, is linked to changes in the composition of the milk in the infected mammary gland (Kelly et al., 2006) (Pisanu et al., 2015).. Particularly during parturition, hormones, metabolites, and acute-phase proteins influence the course of mastitis. According to Paape et al. (2002), the level of circulating PMN in newborn cows has a strong genetic component and is directly related to how susceptible they are to developing clinical mastitis. The altered milk composition can reveal important information about the degree of tissue damage occurring inside the mammary gland as well as the strength of the immune response. Researchers and veterinarians can

make decisions regarding treatment approaches by keeping an eye on these changes to gauge the severity of an illness, evaluate the efficiency of immune responses, and so on.

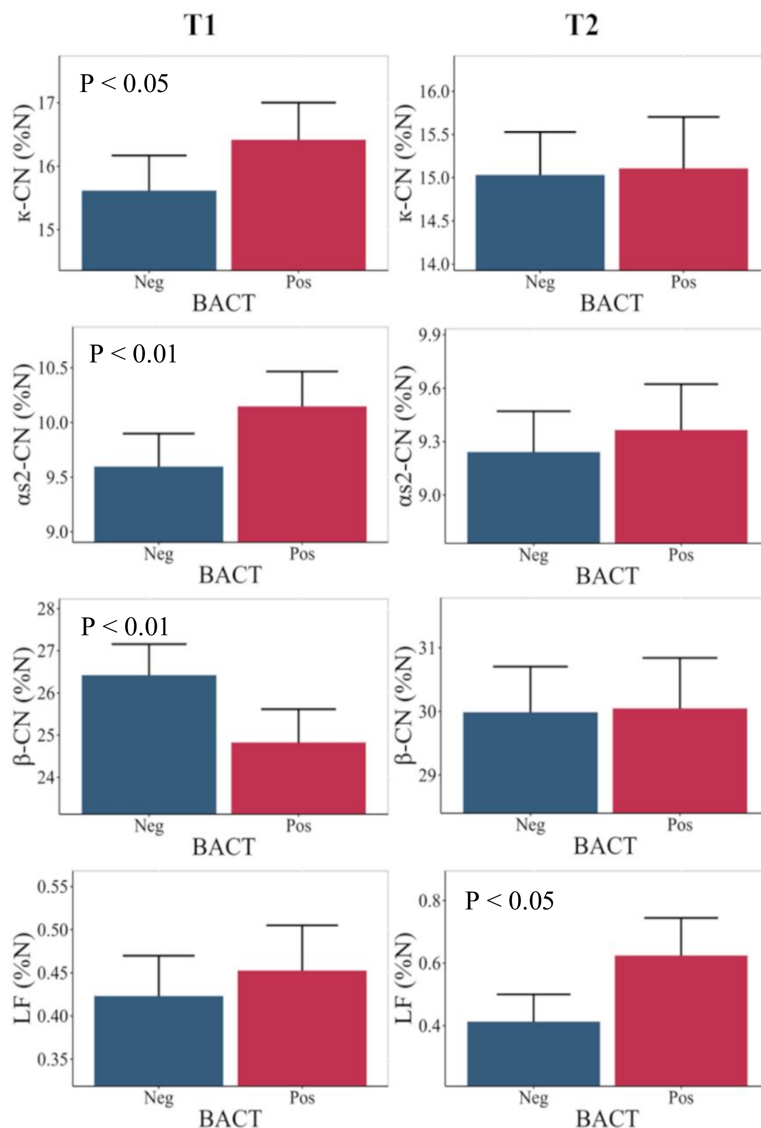


Figure 14. Least square means and standard error (SE) of the bacteriological status on the protein fractions expressed qualitatively (%N) at T1 and T2, respectively. BACT classes were defined as: negative at bacteriological examination (neg) and positive at bacteriological examination (pos). Only significant ($P < 0.05$) traits for at least one sampling time were displayed.

Additionally, bacteria that cause infections have the ability to release outside enzymes into milk, such as elastases (Guerrero et al., 2016), which can aid in the process of proteolysis. This enzymatic degradation seems to affect different protein segments in different ways. For instance, Considine et

al. (2000) discovered that elastase exhibits a specific inclination for cleaving s1-CN and -CN, suggesting that these two may be the most receptive substrates for proteolysis among the casein components. The secretions from mammary glands with clinical mastitis contained elastase activity and bovine lactoferrin (bLF) molecules with low affinity for concanavalin A (Con A). The amount of Con A low affinity bLF present in these secretions from mastitic mammary glands and the level of elastase activity were both shown to increase when visible symptoms changed (Komine et al., 2006). The presence of subclinical intramammary infection (IMI) at the second time point (T2) was associated with increased lactoferrin levels (significant at P 0.05; an increase of 51%; shown in Figure 14). Polymorphonuclear neutrophils (PMN) and glandular epithelial cells both produce lactoferrin, a glycoprotein that binds to iron (Cheng et al., 2008). It is well known that this protein has antibacterial properties. It is well known that this protein has antibacterial properties. According to evidence presented by Chaneton et al. (2008), the mammary gland responds to bacterial infection by increasing lactoferrin secretion in a way that is particular to the kind of pathogen. The researchers also found that infections that trigger a stronger lactoferrin reaction adapt to their environment by acquiring resistance to the protein's antibacterial properties. The higher LF content discovered in our investigation agrees with the figures discovered by Chaneton et al. (2008). During our research, we saw a discernible rise in lactoferrin (LF) in response to the bacteriological condition at the whey proteins. Beyond LF, however, no discernible effect on the whey proteins was seen. Previous studies showed that animals with clinical mastitis had lower levels of the proteins β-lactoglobulin (β-LG) and α-lactalbumin (α-LA) (Hogarth et al., 2004). The reduction was attributed to either a decrease in the production and release of these proteins or to physical damage carried on by pathogen invasion of mammary epithelial cells (McFadden et al., 1988). The disruption of barriers that control the transit of chemicals between blood and milk and the inflammatory damage done to the mammary secretory tissues are both responsible for this decrease (Chaneton et al., 2011). However, two-dimensional gel electrophoresis (2-DE) analysis of whey from cows with subclinical mastitis revealed no diminution in either of these proteins (Baeker et al., 2002). This observation is consistent with the findings of our

research. One could speculate that this result may be related to the nature of the extended infection as well as the variety of infection stages among the research animals. Even though all the animals were fully asymptomatic, the fact that we were unable to determine the precise infection stage may have contributed to the lack of a discernible change in the whey proteins.

Additionally, gaining insight into the precise changes in milk composition might help illuminate the larger physiological effects of intramammary infections. Researchers can learn more about potential effects on calf growth, milk processing, and general herd health by examining how these changes affect the nutritional content of milk. Therefore, understanding the complex relationships between immunological activity, microbial invasion, and milk composition is essential for improving both animal health management and the quality of dairy products.

4.5. Associations between milk somatic cell count and protein fractions

In Table 3, the results of the analysis of variance (ANOVA) for the effect of somatic cell count (SCC) on the variables under investigation are detailed. The SCC showed a significant correlation with each of the casein percentages at the first time point (T1). Notably, an increase in SCC levels caused the concentrations of the two casein fractions that are most prevalent to drop, specifically s1-casein (s1-CN) by 8% (P 0.001) and s-casein (s-CN) by 8% (P 0.01). However, the patterns for κ-casein (κ-CN) and s2-casein (s2-CN) were opposite, showing a correspondingly larger presence with rising SCC, rising by 17% and 4.9%, respectively (as shown in Figure 15). This significant reduction seen in αs1-CN and β-CN, constituting the two most found protein fractions, could potentially be attributed to the surge in milk's endogenous proteases. Leucocyte-produced, blood-milk barrier-altering and plasmin-producing enzymes are all included in this category (L. Forsback et al., 2010; Le Roux et al., 2003). While cathepsin B and D, collagenases, and elastases are abundant in somatic cells' lysosomes, plasmin is the main native milk enzyme (Crudden et al., 2005) present in both healthy and mastitic

situations (Guerrero et al., 2016). These enzymes primarily come into play when inflammation first starts (Le Roux et al., 2003; Wedholm et al., 2008).

The segments most susceptible to enzymatic degradation among all casein fractions are β-CN and αs1-CN (Urech et al., 1999; Ramos et al., 2015). A more noticeable creation of peptides derived from -CN and s1-CN has also been found, according to recent studies on the changes in the peptidome of animals suffering from subclinical mastitis (Guerrero et al., 2016; Addis et al., 2020). These results raise the idea that, in comparison to the other casein fractions, κ-CN may be more resistant to these enzymes' proteolytic activity. In terms of the whey proteins, ß-lactoglobulin (ß-LG) and lactoferrin (LF) were significantly impacted by somatic cell count (SCC) (P 0.05 and P 0.001, respectively; see Figure 16). However, in our analysis, the pattern of variance in ß-LG across the different SCC groups showed an uneven tendency, calling for further research. In addition to the protein fractions, a large drop in the urea proportion was seen concurrent with a sharp rise in SCC (significant at P 0.001, with a drop of 9.6%). Ammonium causes the production of urea in the liver, which is then stablely absorbed from the bloodstream into milk (Hayton et al., 2012).

The absence of rumen-degradable proteins, changes in dry matter intake, or changes in the amount of energy in the feed regimen are just a few examples of management and feeding variables that have an impact on urea concentration (Rezamand et al., 2007). Additionally, previous studies have shown that cows with intramammary infections (IMI) and elevated SCC have lower milk urea proportions (Nyman et al., 2014; Timonen et al., 2017). Despite this, the complex biochemical mechanisms behind this link are still poorly understood, calling for additional in-depth analyses.

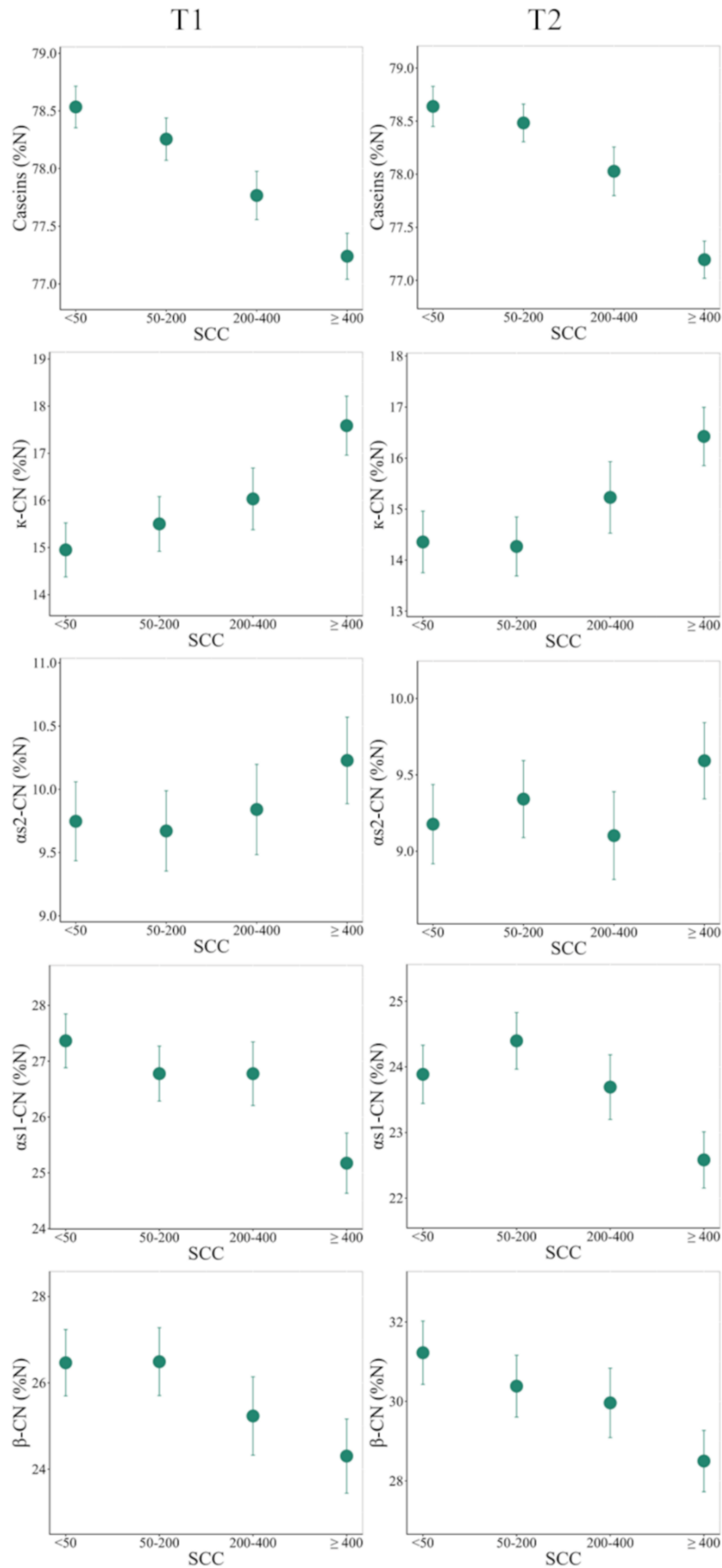
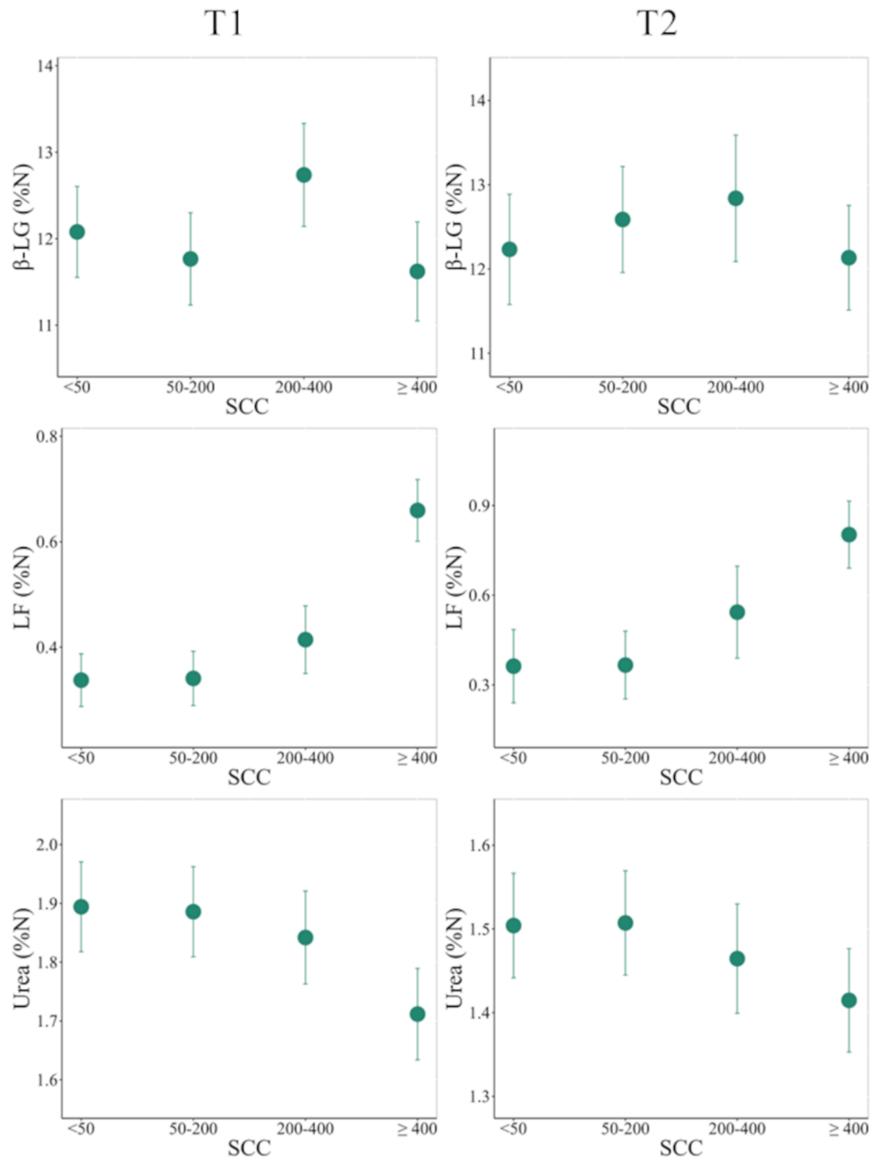


Figure 15. Least square means and standard error (SE) of the SCC classes on the casein fractions expressed qualitatively (%N) at T1 and T2, respectively. Only significant traits ($P < 0.05$) for at least one sampling time were displayed.

Figure 16. Least square means and standard error (SE) of the SCC classes on the whey protein fractions and



milk urea expressed qualitatively (%N) at T1 and T2, respectively. Only significant traits ($P < 0.05$) for at least one sampling time were displayed.

Overall, the two sampling occurrences that were looked at showed consistency in the patterns related to milk protein fractions about various somatic cell count (SCC) classes. To give an example, SCC showed a strong connection with almost all protein fractions at the second time point (T2), except ß-lactoglobulin (ß-LG) and ß-lactalbumin (ß-LA). The levels of ß-casein (ß-CN) were noticeably reduced by 9% (significantly at P 0.001). On the other hand, we confirmed the same pattern seen at

T1 for both κ-casein (κ-CN), which had a considerable rise of 14% (P 0.001), and s2-casein (s2-CN), which saw a slight increase of 5% (P 0.05), where their proportions increased with increasing SCC.

The increase in κ-CN sensitively coincides with the study (Bisutti et al., 2022) that examined the effects of SCC and differential somatic cell count (DSCC) in clinically healthy animals. This behavior's consistency across experiments supports the validity of this phenomenon. The same upward trend also held for lactoferrin (LF) levels, which increased by 121% and were highly significant at P 0.001. Beyond its well-known antibacterial qualities, it's important to remember that LF has also been linked to a variety of immune system consequences. These include both innate and adaptive immune response modification as well as the reduction of inflammatory processes. (Drago-Serrano et al., 2017; Shimazaki and Kawai, 2017). Notably, our analysis revealed a detectable increase in LF concentration from T1 to T2. This behavior might be explained by the continuing development of inflammation. According to de la Rosa et al. (2008), it is possible that the increased generation of LF brought on by the inflammatory state and the enhanced degranulation of polymorphonuclear neutrophils (PMN) both contributed to the buildup of LF. The observed increase in LF concentrations as the inflammatory response progressed over time may be explained by this complicated interplay.

4.6. Association of the interaction between BACT and SCC with milk protein fractions

Intramammary infection (IMI) and somatic cell count (SCC) impacts on the features under inquiry have been thoroughly demonstrated by the results of the analysis of variance (ANOVA) in Table 3. By analyzing the interaction between infection status and the inflammatory process, we were able to concentrate specifically on the significance of subclinical IMI within each SCC class and acquire a clear understanding of its impact. This strategy aims to clarify the key elements causing the alterations in milk protein fractions. The interaction of bacterial presence (BACT) with SCC during the first time

point (T1) revealed a noteworthy relationship with the overall proportion of casein (P 0.01; as shown in Figure 17).

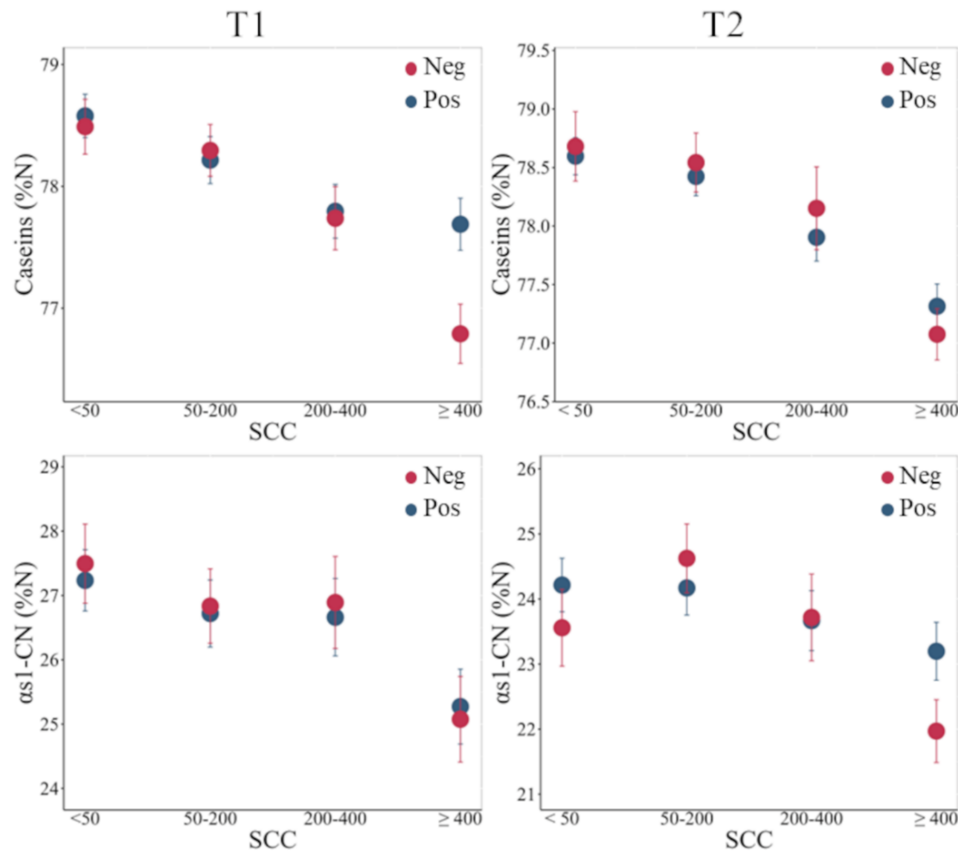


Figure 17. Least square means and standard error (SE) of the bacteriological status (BACT) × somatic cell count (SCC) interaction on the protein fractions expressed qualitatively (%N) at T1 and T2, respectively. Only significant traits (P < 0.05) for at least one sampling time were displayed.

Notably, samples with both positive and negative IMI status showed the highest casein proportions when extremely low SCC (< 50,000 cells/mL), which then showed a gradual decline as SCC values increased. The difference between negative and positive IMI samples became apparent within the SCC class ≥ 400,000, with the latter registering the most decreased casein content (Figure 17).

The presence of bacteria and SCC contact were shown to be related at the second time point (T2) in the context of s1-casein (αs1-CN), (as shown in Figure 17). Specifically, within the positive IMI samples, an increase in αs1-CN was identifiable as the SCC class advanced from the first to the second range (≥ 50,000 and < 200,000 cells/mL), yet this trend was followed by a subsequent reduction, ultimately culminating in its lowest value within the SCC range > 400,000 cells/mL.

Collectively, these results indicate that the influence of IMI on milk caseins seems to be more substantial, especially at significantly elevated SCC levels. Other than the exogenous enzymes that diseases release, some bacteria can stimulate the creation of protease activators. These chemicals, which increase the mammary gland's proteolysis, then release essential substrates that promote bacterial development (Mehrzad et al., 2005; Kelly et al., 2006). This complex dynamic highlight the complex interactions between infection, inflammation, and the ensuing changes in milk protein composition.

5. CONCLUSION

In this study, we wanted to evaluate the effect of naturally occurring subclinical intramammary infection (IMI) and inflammation status (SCC), as well as their interaction, on the detailed milk protein components, at a quarter level. The methodology employed for the identification and quantification of protein fractions through reverse phase chromatography proved to be suitable for processing the large number of samples collected and allowed a good resolution of the major protein fractions (αs1-CN, αs2-CN, β-CN, κ-CN, κ-CN gly, β-LG, α-LA, LF).

The study started by carefully assessing the presence of pathogens across several farms. This was done over two sampling points, T1 and T2, with careful attention to the prevalence and distribution of pathogens. The investigation showed that the protein fraction patterns at the two-time points were similar. The most common microorganism was found to be *S. agalactiae*, which was followed by *Prototheca spp., S. aureus,* and *S. uberis*. Interestingly, no detectable changes in milk production or composition were observed between negative and positive animals during the initial bacteriological examination, suggesting that subclinical infections do not always result in detectable changes. Analysis of the effect of IMI on milk protein fractions demonstrated compelling findings. At T1, there was a strong connection between subclinical IMI and different casein percentages.

In particular, milk quarters with bacterially positive tests exhibited higher levels of κ-CN and s2-CN and lower levels of ß-CN. The activity of immune cells like PMN and macrophages, which release enzymes and cellular components when battling pathogens, was directly tied to these compositional changes. Exogenous enzymes, including the elastases secreted by infected bacteria, were also involved in proteolysis, and had varying effects on various protein fractions. Additionally, lactoferrin (LF), a glycoprotein with known antibacterial capabilities, was found to be upregulated in subclinical IMI at T2. Exogenous enzymes, including the elastases secreted by infected bacteria, were also involved in proteolysis, and had varying effects on various protein fractions. Additionally, lactoferrin (LF), a glycoprotein with known antibacterial capabilities, was found to be upregulated in subclinical

IMI at T2. Interesting findings were found when the link between SCC and protein fractions was examined. SCC and casein fractions displayed a complicated connection at T1. The two casein fractions that are most prevalent, ß-CN and s1-CN, dropped as the SCC increased, but κ-CN and s2-CN displayed the opposite pattern. These variations were ascribed to the disruption of the blood-milk barrier and endogenous proteases secreted because of inflammation, particularly leukocyte enzymes. The complicated impacts of SCC on the protein composition of milk can be seen in the association of whey proteins ß- LG and LF with SCC. Additionally, variations in urea levels were seen, indicating potential metabolic consequences linked to SCC. Further understanding of the subtle influences on milk protein fractions was made possible by considering the relationship between IMI and SCC. At T1, casein content was related to the interplay between bacterial presence and SCC. The samples' varied behavior in the various SCC classes brought this interaction's complexity into sharp relief. At T2, the same interaction began to have an impact on the s1-CN, with varying patterns in the various SCC classes.

Finally, these findings highlight the connections between milk protein fractions, subclinical IMI, and SCC. The most important findings are that IMI and SCC primarily affect caseins (proteolysis), and this effect is more pronounced in positive samples with high SCC and SCC has also been a potent indication of inflammation. The intricate relationships between these variables emphasize the complexity of mammary gland health and milk composition. A deeper knowledge of the dynamics at work in the bovine mammary gland may result from further research that focuses on the fundamental mechanisms driving these interactions.

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