



T.C.

**ÇANAKKALE ONSEKİZ MART UNIVERSITY
SCHOOL OF GRADUATE STUDIES**

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

**INVESTIGATION OF GLYCAN PROFILES OF LACTOFERRINS
FROM DIFFERENT BOVINE BREEDS**

MASTER OF SCIENCE THESIS

RÜYA SESSİZ

Thesis Supervisor

PROF. DR. SERCAN KARAV

ÇANAKKALE – 2025



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ÇANAKKALE ONSEKİZ MART ÜNİVERSİTESİ
LİSANSÜSTÜ EĞİTİM ENSTİTÜSÜ



Rüya SESSİZ tarafından Prof. Dr. Sercan KARAV yönetiminde hazırlanan ve **15/08/2025** tarihinde aşağıdaki jüri karşısında sunulan “**Investigation Of Glycan Profiles Of Lactoferrins From Different Bovine Breeds**” başlıklı çalışma, Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü **Moleküler Biyoloji ve Genetik Anabilim Dalı**’nda **YÜKSEK LİSANS TEZİ** olarak oy birliği ile kabul edilmiştir.

Jüri Üyeleri

İmza

Prof. Dr. Sercan KARAV
(Supervisor)

Prof. Dr. Kemal Melih TAŞKIN

Doç. Dr. Kaan HÜRKAN

.....

.....

.....

Tez No : 10745757

Tez Savunma Tarihi : 15/08/2025

.....

Prof. Dr. Melis ULU DOĞRU

Enstitü Müdürü

...../...../2025

ETİK BEYAN / ETHICAL STATEMENT

Çanakkale Onsekiz Mart Üniversitesi Lisansüstü Eğitim Enstitüsü Tez Yazım Kuralları'na uygun olarak hazırladığım bu tez çalışmasında; tez içinde sunduğum verileri, bilgileri ve dokümanları akademik ve etik kurallar çerçevesinde elde ettiğimi, tüm bilgi, belge, değerlendirme ve sonuçları bilimsel etik ve ahlak kurallarına uygun olarak sunduğumu, tez çalışmasında yararlandığım eserlerin tümüne uygun atıfta bulunarak kaynak gösterdiğimi, kullanılan verilerde herhangi bir değişiklik yapmadığımı, bu tezde sunduğum çalışmanın özgün olduğunu, bildirir, aksi bir durumda aleyhime doğabilecek tüm hak kayıplarını kabullendiğimi taahhüt ve beyan ederim.

In this thesis study, which I have prepared in accordance with the Thesis Writing Rules of Çanakkale Onsekiz Mart University Graduate Education Institute; I have obtained the data, information and documents I have presented in the thesis within the framework of academic and ethical rules, I have presented all information, documents, evaluations and results in accordance with the rules of scientific ethics and morality, I have cited and cited all the works I have used in the thesis, I have not made any changes to the data used, I declare that the work I have presented in this thesis is original, and I accept all loss of rights that may arise against me in the event of otherwise.

Rüya SESSİZ

15/08/2025

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Çanakkale, August 2025

ÖZET

INVESTIGATION OF GLYCAN PROFILES OF LACTOFERRINS FROM DIFFERENT BOVINE BREEDS

Rüya SESSİZ

Çanakkale Onsekiz Mart Üniversitesi

Lisansüstü Eğitim Enstitüsü

Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Tezi

Danışman: Prof. Dr. Sercan KARAV

15/08/2025, 50

Laktoferrin, antimikrobiyal savunma ve bağışıklık düzenlemesi başta olmak üzere çok yönlü biyolojik işlevleriyle bilinen bir glikoproteindir. Laktoferrinin glikozilasyon profili, yapısal özelliklerini ve biyolojik aktivitelerini önemli ölçüde etkilemektedir. Bu çalışmada, Guernsey, Holstein ve Jersey olmak üzere üç farklı sığır ırkına ait doğumun ilk gününde elde edilen kolostrum örneklerinden saflaştırılan laktoferrinin N-glikan yapıları araştırıldı. Laktoferrin, ultrafiltrasyon ve ardından heparin afinite kromatografisi kullanılarak saflaştırıldı; varlığı SDS-PAGE analiziyle doğrulandı. N-glikanlar PNGase F enzimi ile enzimatik olarak serbest bırakıldı ve fenol-sülfürik asit yöntemi ile kantitatif olarak analiz edildi. Enzimatik işlem sonrasında tüm örneklerde glikan içeriğinde artış gözlemlendi; Guernsey (%24,7) ve Jersey (%25,6) örneklerinde bu artış Holstein (%9,6) örneklerine göre daha yüksek bulundu. MALDI-TOF-MS kullanılarak yapılan yapısal analizde Holstein, Guernsey ve Jersey örneklerinde sırasıyla 44, 38 ve 24 farklı glikan yapısı tanımlandı. Guernsey ve Jersey laktoferrinlerinde daha fazla kompleks glikan yapısı bulunurken, Holstein örneklerinde yüksek mannoz tipi glikanların baskın olduğu görüldü. Tüm ırklarda hibrit glikanların düşük oranda tespit edilmesi, örneklerin laktasyonun erken dönemine ait olmasıyla açıklanabilir. Bu bulgular, sığır laktoferrininde ırka bağlı glikozilasyon farklılıklarını ortaya koymakta olup, fonksiyonel süt ürünleri ve biyoterapötik uygulamalar için uygun sığır ırklarının seçiminin önemini vurgulamaktadır.

Anahtar Kelimeler: Kolostrum, Laktoferrin, Glikolizasyon

ABSTRACT

INVESTIGATION OF GLYCAN PROFILES OF LACTOFERRINS FROM DIFFERENT BOVINE BREEDS

Rüya SESSİZ

Çanakkale Onsekiz Mart University

School of Graduate Studies

Master of Science Thesis in Molecular Biology and Genetics

Advisor: Prof. Dr. Sercan KARAV

08/15/2025, 50

Lactoferrin is a multifunctional glycoprotein, particularly involved in antimicrobial defense and immune regulation, whose glycosylation pattern plays an important role in determining its structural and biological characteristics. This present study aimed to investigate the N-glycan composition of lactoferrin isolated from first-day colostrum samples of three different bovine breeds: Guernsey, Holstein, and Jersey. Lactoferrin was purified using ultrafiltration followed by heparin affinity chromatography, and its presence was confirmed through SDS-PAGE analysis. N-glycans were enzymatically released using PNGase F. N-glycans quantified via the phenol-sulfuric acid assay. After enzymatic treatment, a notable increase in glycan content was observed in all samples, with Guernsey (24.7%) and Jersey (25.6%) showing greater increases compared to Holstein (9.6%). Structural characterization using MALDI-TOF-MS revealed 44, 38, and 24 glycan structures in Holstein, Guernsey, and Jersey samples, respectively. While Guernsey and Jersey lactoferrins contained more complex glycans, Holstein lactoferrin predominantly exhibited high-mannose types. Hybrid glycans were found in low abundance across all breeds, likely due to the early stage of lactation from which samples were collected. These findings demonstrate distinct breed-specific glycosylation patterns in bovine lactoferrin and highlight the necessity of considering breed selection in the development of functional dairy products and therapeutic applications.

Keywords: Colostrum, Glycosylation, Lactoferrin



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SYMBOLS AND ABBREVIATIONS

PNGase F	Peptide-N ⁴ -(N-acetyl- β -glucosaminy1) asparagine amidase F
MALDI-MS	Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry
mTOR	Mechanistic target of rapamycin
β -Lg	β -Lactoglobulin
LPO	Lactoperoxidase
LF	Lactoferrin
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
ATP	Adenosine Triphosphate
PTMs	Post-translational modifications
GalNAc	N-acetylgalactosamine
GlcNAc	N-acetylglucosamine
Xyl	Xylose
Fuc	Fucose
Gal	Galactose
Man	mannose
Glc	Glucose
GlcA	Glucuronic acid
IdoA	Iduronic acid
SA	Sialic acid
GPI anchoring	Glypiation
Asn	Asparagine
Ser	Serine
Thr	Threonine
Endo H	Endoglycosidase H
pH	Potential of Hydrogen
NMR	Nuclear magnetic resonance
HCl	Hydrochloric acid
NaCl	Sodium Chloride
rpm	Revolutions per minute
RNase B	Ribonuclease B

2-AA	2-aminobenzoic acid
DMSO	Dimethyl sulfoxide
ACN	Acetonitrile
SDS-PAGE	Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis
OD	Optical density
mg	Miligram
mL	Milliliter
M	mol per litre
nm	Nanometre
μL	Microliter
μg/mL	Microgram per milliliter
TFA	Trifluoroacetic Acid

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CHAPTER ONE

INTRODUCTION

Bovine lactoferrin is versatile glycoprotein found in colostrum and milk, recognized for its broad biological activities, including antiviral, antimicrobial, antioxidant, and immunomodulatory effects (García-Montoya et al., 2012). Colostrum is milk secreted after birth first. It is rich in bioactive components, particularly lactoferrin, which supports the immunity and development of newborns (Uruakpa et al., 2002). The concentration and contents of lactoferrin may depending on factors. Lactation stage, breed, environmental conditions and animals' health are factors that affect the amount of lactoferrin (Adam et al., 2008; Hirai et al., 1990; Jahan et al., 2020; Tsuji et al., 1990). Glycosylation, which occurs particularly with the attachment of carbohydrate chains to protein backbone. Glycans that bind to lactoferrin play an important role in influencing lactoferrin stability, receptor binding, and immune interactions. They may also be involved in regulating lactoferrin's biological functions (Zlatina and Galuska, 2021). Therefore, understanding the species-specific glycosylation patterns of bovine lactoferrin is of great importance to optimize its application in functional dairy products and therapeutics.

Despite its importance, the glycan profiles of bovine lactoferrin from different breeds have not yet been adequately investigated. This knowledge gap limits our understanding of how breed-related glycosylation differences may affect the biofunctional properties of lactoferrin. These differences could be important for making milk-based treatments, creating functional foods for specific bovine breeds, and using lactoferrin in biomedicine and biotechnology. Filling this research gap is important for improving scientific understanding and helping practical uses in industry.

In this study, lactoferrin was first purified from colostrum samples of Jersey, Holstein, and Guernsey breeds. To analyze the glycans, N-glycans were released using the Peptide-N⁴-(N-acetyl- β -glucosaminyl) asparagine amidase F (PNGase F) enzyme. Then, their amount was measured with a colorimetric method, and their structures were studied using MALDI-TOF-MS.

This study aimed to investigate breed-specific differences in the glycan profiles of bovine lactoferrins purified from colostrum samples obtained from Jersey, Holstein, and Guernsey breeds.

The objectives of this thesis were as follows:

- To purify lactoferrin from colostrum samples of Jersey, Holstein and Guernsey bovine breeds.
- To release N-glycans from purified lactoferrin using PNGase F enzyme.
- To quantitatively determine the released N-glycans using a colorimetric assay.
- Characterization of structural differences in N-glycan profiles of lactoferrins obtained from different bovine breeds by MALDI-TOF-MS.

The underlying hypothesis of this study was that:

H0: There is no difference in the glycan profiles of lactoferrins isolated from colostrum of Jersey, Holstein and Guernsey breeds.

H1: There are breed-specific differences in the glycan profiles of lactoferrins isolated from colostrum of Jersey, Holstein and Guernsey breeds.

1.1. Milk and Colostrum

Milk is a unique and complex biological fluid. It secreted by the mammary glands. This unique fluid designed to meet all essential nutritional requirements of the newborn. Milk is a nutrient-dense, animal-derived food characterized by its complex structural matrix. Comprising approximately 87% water, 9% water-soluble compounds (including proteins, lactose, minerals, and vitamins), and 4% lipids, this matrix encompasses both essential nutrients and biologically active components, particularly the proteins casein and whey (Burke et al., 2018).

The period in which mammary secretions in bovine are classified as colostrum until they become mature milk varies considerably in the literature. This period has been reported to range from immediately after parturition (Levieux and Ollier, 1999) to two days postpartum (Playford et al., 2000), three to four days (Davis et al., 2007) or even up to five

to seven days after calving (Georgiev, 2008). The compositional and physicochemical properties of colostrum vary considerably, being influenced by numerous factors such as genetic individuality, parity, breed, nutritional status in late pregnancy, duration of the dry period and time elapsed after parturition (McGrath et al., 2016).

Colostrum is the first milk produced after birth and is characterized by its high nutritional content. It not only provides essential nutrients like fats, proteins, and lactose but also contains immune-supporting compounds such as various immunoglobulins and lactoferrin. In addition, it includes fat-soluble vitamins, such as vitamins A-E, and β -carotene, also water-soluble vitamins, such as thiamine, niacin, vitamin B12, and various forms of vitamin B6. Essential elements (calcium, iron, copper, zinc, sulfur, phosphorus, magnesium, sodium, potassium, and manganese) are also present in significant amounts (Kehoe et al., 2007). Colostrum contains higher amounts of protein, fat, immunoglobulin, minerals and vitamins than mature milk. Because some vitamins cannot cross the placenta, colostrum serves as the newborn's primary source of these essential nutrients after birth (Quigley Iii and Drewry, 1998). Colostrum refers to the early fluid secreted from the mammary gland during the early postpartum period while the spaces between mammary epithelial cells are still open. This allows for the translocation of various immune factors from the maternal bloodstream into the colostrum through paracellular or transcellular pathways. Consequently, colostrum contains elevated levels of secretory immunoglobulin A, lactoferrin, growth factors, antioxidants, and various other bioactive substances with protective and anti-inflammatory properties (Neville, 2001).

1.1.1. Milk Proteins

Milk proteins are recognized as a natural source of bioactive compounds and are widely used in the food industry due to their rich nutritional profile. In this context, milk primarily consists of two major protein groups: casein and whey, both of which contribute to its functional and health-promoting properties (Nayik et al., 2024).

Casein

Among milk proteins, casein is both quantitatively dominant, accounting for up to 80% of the protein in bovine milk, and functionally significant. It is classified as a phosphoprotein, as it contains approximately 0.7–0.9% phosphorus, covalently bound through serine phosphate ester linkages. Its molecular weight ranges from 19 to 25 kDa, and the isoelectric point varies between pH 4.1 and 5.3. All casein fractions are amphipathic in nature, a property that facilitates the formation of micellar structures in the presence of phosphate and calcium ions (Broyard and Gaucheron, 2015).

Casein consists of four primary fractions: α s1-casein, which makes up about 40%; β -casein, around 35%; κ -casein, approximately 15%; and α s2-casein, which accounts for roughly 10%. These subtypes are water-insoluble and aggregate with colloidal calcium phosphate to form large, spherical micelles, which play a critical role in the stability, functionality, and digestibility of milk (Bhat et al., 2016).

Whey Proteins

Whey proteins are also called serum proteins. It constitutes the soluble protein fraction of milk that remains in the liquid phase after casein has precipitated during acidification or enzymatic coagulation. These proteins are predominantly globular in structure and compose approximately 20% of the total protein content in bovine milk. Owing to their rich nutrient composition, excellent digestibility, and wide range of functional characteristics, whey proteins have attracted significant attention in both food technology and nutritional science (Kilara and Vaghela, 2018). Whey protein has been widely studied for its benefits on muscle health. It supports muscle growth by activating the mechanistic target of rapamycin (mTOR) pathway, which controls cell growth and protein production. mTOR is responsible for integrating signals from hormones, energy status, physical activity, and amino acid concentrations. Leucine serves as the primary trigger of mTOR activation in adults. Whey protein, which contains approximately 11–12% leucine, reaches this threshold more efficiently than other protein sources, enabling muscle protein synthesis with less protein and fewer calories. Whey protein is particularly beneficial in situations such as post-surgery or illness recovery, athletic training, or dietary interventions for individuals with increased protein needs (Auestad and Layman, 2021). Whey proteins are made up of several

important components. The two most abundant proteins in whey are β -lactoglobulin and α -lactalbumin. In addition to these, whey also contains other bioactive proteins. These include immunoglobulins, bovine serum albumin, and lactoferrin. Each of these proteins plays important roles in immune defense, mineral binding, and antioxidant activity, contributing uniquely to the nutritional and functional properties of whey (Kilara and Vaghela, 2018; Nayik et al., 2024).

β -Lactoglobulin

β -Lactoglobulin (β -Lg) is the most abundant protein in the whey portion of bovine milk. It accounting for approximately 50–58% of total whey proteins and approximately 10% of total milk protein (Chatterton et al., 2006). It is either missing or found in very small amounts in human milk, but it is found in large amounts in ruminant milk. β -Lg is considered a potential allergen, particularly in individuals with milk protein sensitivity (Wei et al., 2018). β -Lg is a globular protein composed of 162 amino acids. β -Lg has a molecular weight of approximately 18 kDa and an isoelectric point between 5.1 and 5.2. It remains stable under acidic conditions but is heat-sensitive and denatures upon heating. In milk, β -Lg exists primarily in the dimeric form and exhibits high solubility and gel-forming capacity over a wide pH range of 3 to 7 (Chatterton et al., 2006). Although the function of β -Lg in the body has not yet been clearly explained, it is believed to help carry fat-soluble substances. Furthermore, its rapid digestibility, functional properties, and high biological value have made it a widely used protein source in dietary supplements, sports nutrition, and protein-enriched beverages (Kontopidis et al., 2004). In addition to its nutritional value, varied bioactive peptides obtained from the proteolytic digestion of β -Lg have been reported to exhibit diverse biological activities. These include angiotensin-converting enzyme inhibitory effects, antimicrobial activity, and inhibition of pathogen adhesion. These functions have been demonstrated with studies, emphasizing that β -Lg may be a functional food ingredient (Auestad and Layman, 2021; Chatterton et al., 2006).

α -Lactalbumin

α -Lactalbumin makes up approximately 20-25% of the total whey proteins, which ranks it as the second most prevalent protein in whey. It is characterized by its calcium-

binding capacity, heat stability, and non-gelation (Yadav et al., 2015). α -Lactalbumin has an isoelectric point of 4.2 and a molecular weight of approximately 14 kDa. Its rich amino acid composition, which includes nearly all essential amino acids as exemplified by aspartic acid, leucine, isoleucine, valine, and sulfur-containing amino acids, makes α -lactalbumin a nutritionally valuable protein (Guo and Wang, 2019).

This protein's high tryptophan content may contribute to increasing brain serotonin levels, helping to reduce stress and depression and improve sleep quality. Given that tryptophan serves as a direct precursor for serotonin synthesis, the mood-regulating and sleep-promoting effects of α -lactalbumin have attracted scientific interest. In addition to tryptophan, its high cysteine content supports the synthesis of biologically important antioxidants such as glutathione and taurine. The disulfide bridges found in α -lactalbumin help maintain the protein's tertiary conformation, enabling it to exhibit prebiotic-like biological activities. Furthermore, α -lactalbumin plays many physiological roles in the gastrointestinal tract, including supporting cell growth, contributing to antimicrobial defense, and enhancing motility (Auestad and Layman, 2021).

Immunoglobulins

Immunoglobulins belong to the globular protein family and exist at a significantly high concentration of approximately 6% in colostrum, while their content is reduced to roughly 0.1% in mature milk. The high globulin content in colostrum contributes to heat-induced coagulation, a property not observed in normal milk due to its significantly lower globulin concentration. Immunoglobulins have a molecular weight between 80 and 800 kDa (Nayik et al., 2024).

Immunoglobulins have high nutritional value and contain all essential amino acids. Immunoglobulins have a key function in passive immunity. Numerous studies have demonstrated that immunoglobulins derived from colostrum protect against microbial infections (Hurley and Theil, 2011).

Serum Albumin

Serum albumin is a water-soluble, milk-derived protein with a molecular weight of approximately 66.5 kDa and an isoelectric point of 4.7. It consists of a single polypeptide chain of 583 amino acids. Serum albumin constitutes approximately 5–10% of the total whey protein content (Nayik et al., 2024). The presence of amino acids such as methionine, isoleucine, valine, leucine, tyrosine, and proline within the protein structure significantly contributes to its biological activity (Tovar-Jiménez et al., 2017). Serum albumin is a potent source of bioactive peptides with significant physiological and pharmacological effects. Its antioxidant properties, such as Neutralizing free radicals, preventing lipid peroxidation, and chelating metal ions, are relevant to the prevention of chronic degenerative diseases (Ballatore et al., 2020).

Lactoperoxidase

Lactoperoxidase (LPO) is a peroxidase enzyme naturally found and secreted into the salivary, mammary, and lacrimal glands of mammals. LPO is a glycoprotein composed of 612 amino acids with a molecular weight of approximately 78 kDa and a carbohydrate content of 8–10% (Rombauts et al., 1967). LPO constitutes approximately 0.5% of the total whey protein content and is present at a level of approximately 30 mg/L in bovine milk whey (De Wit and Van Hooydonk, 1996). LPO catalyzes the oxidation of thiocyanate with hydrogen peroxide to produce the antimicrobial hypothiocyanide, contributing to the natural defenses of secretions such as saliva and milk. Its broad regulatory and antimicrobial effects, coupled with its role as a natural preservative, offer potential for therapeutic or preventative use (Özhan et al., 2025).

Lactoferrin

Lactoferrin (LF), also called lactotransferrin, belongs to the transferrin family with approximately 60% sequence similarity to serum transferrin and is a basic, positively charged glycoprotein weighing approximately 80 kDa. LF has a strong ability to bind and transport Fe^{3+} ions (Metz-Boutigue et al., 1984). The LF gene is expressed in a lot of species, including humans, cattle (cows and buffalo), pigs, horses, goats, deer, and mice (Dyrda-

Terniuk and Pomastowski, 2023). The salmon pink color of lactoferrin is characteristic and is due to the ferric ions present in its structure (Lönnerdal and Iyer, 1995). LF three-dimensional structure and amino acid sequence vary among mammalian species. For example, bovine lactoferrin is a single polypeptide chain of 689 amino acids, while this number is 691 in human lactoferrin. Bovine and human lactoferrin share approximately 68% amino acid sequence identity (Gruden and Poklar Ulrih, 2021). Each molecule has two globular lobes, called the C-terminal and N-terminal lobes, connected by α -helical regions; each lobe is further divided into two subregions (Anderson et al., 1987). In the iron-bound (holo) form, both lobes are locked in a closed conformation. In its iron-free (apo) form, the N-terminal lobe of lactoferrin typically adopts an open conformation, indicating the absence of coordinated iron within its binding site. In contrast, the C-terminal lobe displays greater structural flexibility, as it is capable of existing in either an open or closed conformation depending on the local molecular environment (Brisson et al., 2007).

Lactoferrin has many different biological functions. First, it sequesters Fe^{3+} in microbial environments in its iron-free (apo) form, depriving pathogens of this essential nutrient, thus exerting a bacteriostatic effect. Furthermore, its positively charged N-terminal region provides bactericidal activity by binding directly to bacterial cell wall components including lipopolysaccharide and lipoteichoic acid. Lactoferrin also provides antiviral protection by preventing the entry of various DNA and RNA viruses into host cells. It acts against fungal pathogens by both restricting iron and permeabilizing the cell surface, while it supports defense against protozoa and other parasites by disrupting the cell membrane or inhibiting intracellular growth. In addition to these antimicrobial roles, lactoferrin regulates both innate and adaptive immunity: it suppresses the release of proinflammatory cytokines, promotes the maturation of antigen-presenting cells, and enhances the activation of natural killer cells and polymorphonuclear leukocytes. Lactoferrin has been shown to induce cell cycle arrest and promote apoptosis in various cancer cell types. Through these mechanisms, it disrupts the uncontrolled proliferation of malignant cells and contributes to the suppression of tumor growth. As a result, lactoferrin is recognized for its antitumor properties and potential therapeutic applications in cancer treatment. LF further expands its functional repertoire by exhibiting various enzymatic activities, including phospholipase, DNase, RNase, and ATPase (González-Chávez et al., 2009).

1.1.2. Purification Methods of Proteins

To elucidate the biological functions of proteins and enable their use in research or industrial processes, it is essential that they be obtained in high purity. Protein purification is a multistep process that involves isolating a specific protein from complex biological mixtures by exploiting its physical, biological, and chemical properties (Konak et al., 2014). Achieving desired purity of a single protein requires multiple purification steps. To successfully isolate a specific protein from a mixture, it's essential to understand its unique physical and chemical properties. Then, it's essential to select appropriate purification methods based on the protein being isolated. There is no universal or simple method that applies to purifying all proteins. Furthermore, the procedures and conditions used to purify one protein may result in the degradation or inactivation of another protein (Hedhammar et al., 2006).

Membrane Filtration Methods

Membrane filtration is a physical separation technique. This technique allows fractionation of whey components based on particle size. Particles that are small enough to pass through the membrane are called permeate. Those that are too large stay on the surface and form the retentate, which is the concentrated part. Since membrane filtration is performed without the use of heat, it prevents structural changes in whey proteins that can be caused by heat and the degradation of heat-sensitive micronutrients such as vitamins (Wen-Qiong et al., 2019)

Ultrafiltration is an effective membrane-based technique for obtaining protein from whey with high purity and yield. Using membranes with different molecular weight cutoffs, proteins can be separated based on their molecular size. To prevent membrane fouling during ultrafiltration, microfiltration is often performed beforehand to remove suspended fat globules and casein micelles. Ultrafiltration membranes generally allow particles in the range of approximately 0.01–0.1 μm to pass. During the ultrafiltration process, small molecules such as lactose and minerals pass through the membrane, while larger structures e.g. whey proteins accumulate in the retentate. This results in a permeate rich in minerals and lactose and a retentate rich in proteins. To increase the protein concentration, the

retentate can be diluted with deionized water and subjected to a second ultrafiltration step (Maubois, 2011).

Microfiltration is one of the oldest methods among membrane separation technologies and operates with pore sizes in the range of approximately 0.01–10 μm , providing a separation capacity between coarse filtration and ultrafiltration (Huppertz et al., 2017). In dairy processing, ceramic membranes are often used for microfiltration because they can withstand high heat and a wide range of pH levels. Microfiltration is typically performed at 50–55°C, under transmembrane pressures of 0.1–1.0 bar, and with tangential flow velocities exceeding 6 m/s. Whey obtained through microfiltration contains higher levels of caseinomacropptides than whey obtained by conventional separation methods (Tolkach and Kulozik, 2005).

Nanofiltration is a membrane filtration technique designed to retain smaller particles, typically in the 0.001–0.01 μm range. During this process, salts, ions, and a small amount of water pass through the membrane, while macromolecules such as proteins remain in the retentate. Lactic acid is also removed during nanofiltration, resulting in a demineralized and concentrated whey product free of salty or sour flavors. The resulting permeate is rich in minerals, ions, and water and can be reused as a mineral supplement in animal feed or for industrial cleaning implementations (Belleville et al., 2021).

Another effective membrane-based strategy for concentrating whey components is reverse osmosis. Reverse osmosis membranes operate in the 0.0001–0.001 μm range, retaining virtually all solutes while allowing only water molecules to pass through. Consequently, all whey components are concentrated in the retentate, while the permeate consists solely of pure water (Marx et al., 2018).

Chromatographic Methods

Chromatographic techniques commonly employed in protein purification. Affinity chromatography, ion exchange chromatography, hydrophobic interaction chromatography and gel filtration chromatography are the most frequently preferred chromatographic methods. These purification techniques take advantage of various properties of proteins,

including their solubility, electrical charge, affinity for water or non-polar environments, size, or ability to bind to specific biological molecules. The protein purification process typically does not rely on a single method but rather involves the sequential application of multiple chromatographic steps. These methods complement one another to ensure that the target protein is obtained with the desired level of purity while preserving its biological activity. However, in applications requiring high specificity and selectivity, affinity chromatography has emerged as a preferred technique due to its exceptional selectivity, operational efficiency, and ability to achieve high purity in a single step (Konak et al., 2014).

Affinity chromatography is considered the most selective chromatographic technique employed in protein purification processes, often enabling the isolation of proteins at high purity in a single step (Doonan and Cutler, 2004). This method is particularly effective for the purification of biologically significant molecules such as enzymes, hormones, antibodies, and glycoproteins (Hage, 2006). In affinity chromatography, the target protein binds specifically and reversibly to a ligand fixed on a solid surface, allowing selective separation. The binding between the ligand and the protein is usually governed by non-covalent interactions such as electrostatic attractions, hydrogen bonds, van der Waals forces, or hydrophobic effects. After the target protein binds to the immobilized ligand, it can be specifically released by changing conditions of the mobile phase, such as adjusting the pH, salt level, or ionic strength. Commonly used ligands in this technique include antibodies, enzyme inhibitors, lectins, metal chelates, and nucleic acids. The solid supports employed for ligand immobilization are generally based on agarose, dextran, or cellulose matrices, which offer suitable mechanical stability and biocompatibility for chromatographic applications (Konak et al., 2014). Because heparin carries a strong negative charge and binds specifically to certain proteins, it has been effectively used to isolate basic proteins from whey, especially those found in small amounts (Ounis et al., 2008).

Ion exchange chromatography separates proteins by relying on variations in protein's surface charge characteristics. By selecting an appropriate pH and ionic environment relative to the proteins' isoelectric points, positively or negatively charged proteins can be selectively bound to oppositely charged resins. This technique is particularly favored during initial purification steps due to its productive binding capacity and economic efficiency (Selkirk, 2004).

Hydrophobic interaction chromatography is relied on the interaction between immobilized hydrophobic ligands on the chromatographic matrix and non-polar regions on the surface of proteins. This method is particularly useful when it is essential to preserve the native three-dimensional structure of proteins during purification. Hydrophobic interactions are promoted by the presence of high ionic strength salts, and elution is typically accomplished by gradually decreasing the salt concentration (Konak et al., 2014). Hydrophobic interaction chromatography is commonly employed for the separation of partially hydrophobic proteins, such as serum proteins and membrane proteins (Lienqueo et al., 2003).

Gel filtration chromatography, also called size exclusion chromatography, separates proteins based on their size. Smaller molecules enter the pores of the gel and take longer to pass through, while larger ones bypass the pores and come out earlier. This technique is primarily used as a final purification step for the removal of aggregates or contaminants, for molecular weight estimation, and for buffer exchange (Konak et al., 2014).

1.2. Proteins Posttranslational Modifications

Post-translational modifications (PTMs) are covalent changes to a protein's backbone or side chains that increase chemical diversity beyond the twenty standard amino acids (Müller, 2018). By allowing rapid and reversible alterations, PTMs enable dynamic regulation of proteins in development and physiological responses without relying on slower genetic changes. So far, researchers have identified more than 650 different types of PTMs, including well-known ones like phosphorylation, acetylation, glycosylation, methylation, and ubiquitination as well as others such as acylation, cysteine oxidation, SUMOylation, ADP-ribosylation, and citrullination. New PTMs are still being discovered. This wide range of covalent changes produces a large variety of proteoforms, which underlie complex cellular functions and organismal adaptation (Zhong et al., 2023).

Proteins can undergo post-translational modifications at several phases of their functional life. Some proteins are modified right after they are made to help them fold properly, whereas others, especially stable ones, can be altered later in response to signals,

either activating or inhibiting downstream pathways (Ahearn et al., 2012). PTMs influence protein behavior by altering their structure, function, charge, stability, and ability to interact with other molecules such as DNA, RNA, or proteins across and within cells. These changes can ultimately shape cellular phenotypes and affect numerous biological functions. PTMs play key roles in a wide range of cellular processes, including the regulation of the cell cycle, differentiation, transcription, metabolism, immune defense, signaling pathways, and autophagy. When these modifications occur abnormally, they can disrupt protein function and contribute directly to the development and progression of various diseases (Zhong et al., 2023).

As the most prevalent form of post-translational modification, glycosylation gives rise to glycans, which are vital macromolecules found in every form of life (Varki, 2017).

1.2.1. Glycosylation

Protein glycosylation refers to the enzymatic addition of sugar moieties to proteins, a process that mainly occurs in the endoplasmic reticulum and Golgi apparatus with the help of glycosyltransferases. In contrast, glycosylation events in the nucleus and cytoplasm tend to involve simpler structures and are highly dynamic. Most glycans are built from a core set of ten monosaccharides, including N-acetylglucosamine (GlcNAc), galactose (Gal), mannose (Man), sialic acid (SA), glucose (Glc), fucose (Fuc), xylose (Xyl), N-acetylgalactosamine (GalNAc), glucuronic acid (GlcA), and iduronic acid (IdoA). Protein glycosylation occurs mainly through four mechanisms: N-glycosylation, where sugars attach to asparagine residues; O-glycosylation, which involves serine, threonine, or tyrosine residues; C-mannosylation, targeting tryptophan; and glypiation, where glycosylphosphatidylinositol (GPI) anchors link certain proteins to cell membranes. These modifications are essential for controlling the activity, stability, and localization of both intracellular and secreted proteins. They also play an important role in supporting a wide range of biological functions within the human body, including processes related to immunity, cellular growth, metabolism, and overall physiological regulation (Zhong et al., 2023).

N-glycans are linked to proteins through a covalent bond between the sugar chain and the asparagine (Asn) residue, forming an N-glycosidic connection. In eukaryotic systems, this linkage consistently starts with N-acetylglucosamine (GlcNAc) attached to Asn in a $\beta 1$ configuration. Although prokaryotes also perform N-glycosylation, they often use a wider variety of sugar units. The consensus sequence required for this modification is Asn-X-Ser/Thr, where 'X' represents any amino acid except proline. The core structure of eukaryotic N-glycans is conserved and consists of Man α 1-3(Man α 1-6) Man β 1-4GlcNAc β 1-4GlcNAc β 1-linked to Asn in the Asn-X-Ser/Thr motif. Depending on how this core is modified, N-glycans are divided into three major types (Figure 1):

- Oligomannose: The core is extended exclusively by mannose residues.
- Hybrid: The Man α 1-6 arm is extended with mannose sugars, while the Man α 1-3 arm contains one or two GlcNAc-initiated antennae.
- Complex: The core branches are extended by one or more antennae, which are initiated by GlcNAc (Stanley et al., 2022).

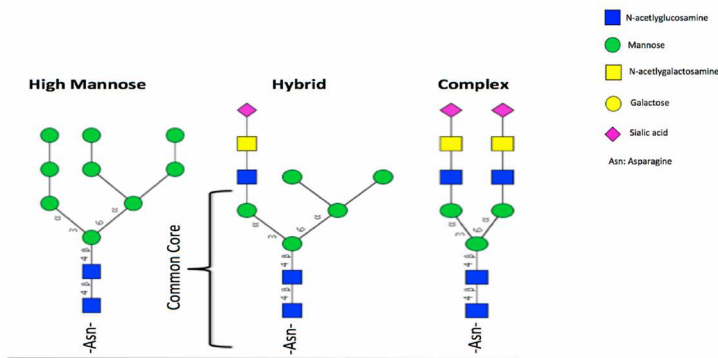


Figure 1. Types of N-glycans (Karav et al., 2017).

N-glycans influence many aspects of glycoprotein behavior, including folding and overall conformation, solubility, enzymatic activity, antigenic properties, and recognition by glycan-binding proteins. These effects make N-glycosylation a key determinant of protein function and interactions (Stanley et al., 2022).

Lactoferrin, like all proteins, undergoes post-translational modifications. Bovine lactoferrin contains five potential N-glycosylation sites, which is two more than human lactoferrin. Asparagine at positions 233, 368, 476 and 545 is fully glycosylated, while Asn281 is only partially modified (Spik et al., 1994). In bovine colostrum, about 30 percent

of lactoferrin molecules carry N-glycans at Asn281, compared with only about 15 percent in mature milk (Zlatina and Galuska, 2021). The C lobe contained a greater number of glycan chains than the N lobe, with three glycans attached to the C lobe. The glycans of lactoferrin are thought to be more complex and heterogeneous than those of other transferrin's. This complexity and the presence of heterogeneous glycans are thought to account for at least some of the differences in the biological properties of lactoferrin (Metz-Boutigue et al., 1984).

Glycan Release Strategies

Deglycosylation methods are essential for producing and studying free glycans while preserving their native structures. These methods fall into two main categories: enzymatic and chemical. These methods may be used alone or in combination to achieve efficient release of O- and N-linked glycans. Hydrazine treatment is among the commonly used chemical methods for deglycosylation. Using this approach, both N- and O-linked glycans can be specifically cleaved from purified glycoprotein preparations. However, due to its carcinogenic and toxic properties, hydrazine is not considered appropriate for food-related or clinical applications (Karav et al., 2017). Chemical methods are faster and more cost-effective compared to enzymatic approaches, yet they carry a higher risk of compromising the structural integrity of glycans and degradation of the protein (Mulloy et al., 2009).

Enzymatic approaches offer a significant advantage in glycan analysis by preserving the integrity of the glycoprotein, allowing for further structural or functional studies. These methods employ specific enzymes to release N-linked glycans without degrading the protein backbone. As an example, PNGase F breaks the bond connecting the core GlcNAc to asparagine, making it possible to release most N-glycans (Tarentino et al. , 1985). Although the released glycosylamine is rapidly hydrolyzed to a hydroxyl form, the structural integrity of the glycan is retained. Another example, Endoglycosidase H (Endo H), specifically cleaves within the chitobiose core of high-mannose and hybrid-type glycans, between two GlcNAc residues (Trimble and Maley, 1984). Neuraminidase and β -galactosidase, which trim the terminal structures of complex and hybrid N-glycans (Skropeta, 2009).

PNGase F (PNGase F from *Falvobacterium meningosepticum* is commonly recognized as the most reliable enzyme for releasing N-linked glycans. It cleaves both high-mannose and complex oligosaccharides with high efficiency. Because it works at neutral pH and moderate temperature (around 37 °C), it preserves protein structure during deglycosylation. The released glycans are obtained in high purity, which improves the sensitivity and reproducibility of downstream mass spectrometry and chromatography analyses. PNGase F's combination of broad substrate specificity, gentle reaction conditions, and consistent performance makes it indispensable for glycan characterization studies (Palm and Novotny, 2005; Tarentino et al., 1985). However, α 1 $\rightarrow$ 3-linked fucose on the innermost GlcNAc inhibits enzymatic activity of PNGase F. Understanding this limitation not only prevents misinterpretation of results but also allows researchers to use it as a selective and strategic tool in glycan analysis (Altmann et al., 1995).

Glycan Characterization via Mass Spectrometry

Mass spectrometry (MS), glycan sequencing, and nuclear magnetic resonance (NMR) spectroscopy are the three main analytical techniques commonly used in glycan analysis (Tekin and Gurbanov, 2024). Among various analytical techniques developed for glycan analysis, MS has become an essential and widely used tool, due to its high sensitivity, specificity, and ability to provide detailed information on glycan mass, composition, and structure. It is often combined with glycan labeling methods and involves steps: sample preparation, separation, ionization, and mass detection. MS is a technique that identifies ions by measuring their mass-to-charge (m/z) ratios. The output, known as a mass spectrum, displays the relative intensity of each ion in relation to its m/z value (Han and Costello, 2013). MS techniques generally offer low detection limits, typically in the femtomole to picomole range (10^{-15} to 10^{-12} mol), although sensitivity may vary depending on ionization efficiency, instrument resolution, and sample complexity. The analysis of mass spectrometry data often relies on dedicated software tools, while strict quality control measures are necessary to maintain accuracy and reproducibility (Tekin and Gurbanov, 2024).

CHAPTER TWO

PREVIOUS STUDIES

Lactoferrin, as a key bioactive protein naturally present in milk, has attracted considerable attention in scientific research. Lactoferrin is found in higher concentrations in colostrum than in mature milk. A study on porcine milk showed that it contains a significant amount of lactoferrin, with the highest concentration found in colostrum. This level decreased by approximately 62% in transitional milk and by around 67% in mature milk (Jahan et al., 2020). In another study conducted in porcine showed that lactoferrin levels were high during the first three days after birth, followed by a sharp decline (Elliot et al., 1984). In a study conducted in cattle, the highest lactoferrin content was detected at 24 hours after birth (Adam et al., 2008). In contrast, a study conducted in cows showed that the mean milk lactoferrin concentration tended to be higher in the late lactation period compared to the peak and middle periods (Cheng et al., 2008). The concentration of lactoferrin in milk exhibits variation across different bovine breeds. In a study examining the breed-related differences in colostrum lactoferrin levels, Jersey cows were shown to have higher lactoferrin concentrations compared to Holstein, Japanese Black, and Japanese Brown breeds (Tsuji et al., 1990). In a study analyzing lactoferrin levels in daily milk samples, Jersey cows were found to have higher lactoferrin content compared to Montbéliarde, Brown Swiss, Belgian Blue, Non-Holstein Red and White, and Normande breeds (Soyeurt et al., 2007).

Characterizing the N-glycosylation patterns of milk proteins should be considered in studies aimed at improving the nutritional and functional properties of milk. It is also important for identifying and developing biomarkers related to animal health and productivity (Guan et al., 2022). The degree of glycosylation of milk varies according to the lactation stages; for instance, it is approximately 30% in colostrum and declines to around 15% in mature milk (Figueroa-Lozano et al., 2018). When focusing specifically on lactoferrin, studies have shown that factors such as the stage of lactation, the animal's age and diet, seasonal variations, and daily milk yield can influence not only the concentration of lactoferrin but also the structure of its glycans (Karav et al., 2017). A study investigating the N-glycan profile of bovine lactoferrin throughout lactation identified 32, 24, and 20 distinct N-glycans in bovine colostrum, transitional milk, and mature milk, respectively. The

findings also indicated a progressive increase in the relative abundance of high-mannose N-glycans, whereas levels of sialylated and fucosylated N-glycans initially increased and then declined as lactation progressed (Jia et al., 2021). The same researchers reported notable variations in the molecular weight of bovine lactoferrin throughout lactation period. They suggested that these differences might be related to changes in protein glycosylation (Jia et al., 2021).

Based on the literature, lactoferrin was purified from day one colostrum of different bovine breeds and their N-glycan structures were compared. The purified lactoferrins treated with PNGase F, and the resulting glycan profiles analyzed to identify differences in glycan composition among the breeds.

CHAPTER THREE

MATERIALS AND METHODS

3.1. Materials

This subsection provides a comprehensive overview of all materials employed throughout the experimental procedures described in this thesis. It includes detailed information on the biological substrates, enzymes, chemicals, as well as the laboratory instruments and equipment essential for the successful execution of each experimental step.

3.1.1. Substrates

Bovine colostrum samples representing various breeds were collected from Uluova Milk Trading Co., a regional dairy supplier based in Çanakkale, Türkiye.

3.1.2. Chemical Reagents, Analytical Kits, and Associated Laboratory Materials

The chemicals, kits, and essential materials required for this study are summarized in Table 1.

Table 1.

List of chemicals, kits, and essential materials.

Material Name	Manufacturer/ Catalog Number	Intended Use
Ethanol	Isolab Laborgeräte GmbH/ 920.026.2500	Protein isolation
Hydrochloric acid (37%)	Merck Millipore/ 100314	For precipitation of residual casein micelles, Affinity Chromatography running buffer

Table 1 continued		
Acetic acid	Merck (Sigma-Aldrich)/137130	
Methanol	Merck (Sigma-Aldrich)/106012	
Coomassie Brilliant Blue R 250	Amresco/ 0472-25G	
SureCast™ Acrylamide Solution (40%)	Thermo Fisher Scientific/ HC2040	
Isopropanol	Isolab Laborgeräte GmbH/ 961.023.2500	
Sodium dodecyl sulfate	BioShop/ SDS001.500	
10X Tris-Glycine-SDS Running Buffer	Bio-Rad/ 1610732	
Laemmli Sample Buffer (2X)	Bio-Rad/ LSB-2x	SDS-PAGE analysis
Tris-base	BioShop/ TRS001.1	
SureCast™ TEMED	Thermo Fisher Scientific/ HC2006	
Ammonium Persulfate	Thermo Fisher Scientific/ 17874	
PageRuler™ Plus Prestained Protein Ladder	Thermo Fisher Scientific/ 26619	
Qubit™ Protein Assay Kit	Thermo Fisher Scientific/ Q33211	Determination of protein concentration
50 kDa Ultra Centrifugal Filter Unit	Merck Millipore (Amicon)/UFC9050	Concentration and buffer exchange of protein samples by centrifugation
Heparin-Sepharose beads	Abcam (Cambridge, UK)/ ab193268	Affinity purification of heparin-binding proteins

Table 1 continued		
Sodium Chloride	Merck Millipore/ 1.06404	Affinity chromatography running buffer
Tween 20	Sigma-Aldrich/ P9416	Affinity chromatography running buffer
10 mL Disposable Syringe	BD/ 309604	For affinity chromatography column
HEPES	Sigma-Aldrich / H3375	Buffer exchange solution
PNGase F	New England Biolabs/ P0704S	Deglycosylation of glycoprotein samples
Glycobuffer 2	New England Biolabs/ P0704S	Buffer for PNGase F digestion of N-linked glycans
Glycoprotein Denaturing Buffer	New England Biolabs/ P0704S	Protein denaturation prior to PNGase F digestion
NP-40 (Nonidet P-40 Substitute)	New England Biolabs/ P0704S	Added to deglycosylation mix to counteract SDS inhibition
RNase B	New England Biolabs/ P7817S	Positive control for PNGase F deglycosylation assays
Sulfuric acid (95-98%)	Merck Millipore/ 112080	Phenol sulfuric acid assay

3.1.3. Laboratory Equipment Used for Molecular Analysis

Table 2 provides an overview of the laboratory equipment used.

Table 2.

List of laboratories equipment.

Equipment Name	Brand name
Cooling centrifuge	Hettich Mikro 200 R
Centrifuge	Beckman Allegra X-15R
Speedvac evaporator	Labconco

Table 2 continued	
Orbital shaker	STUART
Dry bath	Benchmark Scientific BSH1002-E
Thermometer-Portable	IsoLab
pH meter	IsoLab
Ice generator	Izmak
Autoclave	NÜVE
-20°C freezer	Arçelik
Qubit™ 3 Fluorometer	Invitrogen
Vortex	Vortex Genie 2
Incubator	Indem Nüve EN 400
Thermal shaking incubator	INOVA
Pure water system	Millipore, Germany
Microplate Reader	Infinite M200 Pro NanoQuant
Analytical balance (mg)	Shimadzu Corporation (Japan)
Analytical balance (g)	Denver Instrument Company, USA
SDS–PAGE gel documentation system	iBright™ 750 Imaging System (Thermo Fisher Scientific, USA)

3.2. Methods

In this subsection, the experimental procedures adopted for this study are outlined, and the applied methodologies are described in detail.

3.2.1. Protein Isolation from milk samples

Colostrum samples for this study were obtained from a regional dairy farm in Çanakkale (Uluova Milk Trading Co.). The samples, collected from each bovine breed on the first day postpartum, were stored in 100 mL sterile bottles (Figure 2).

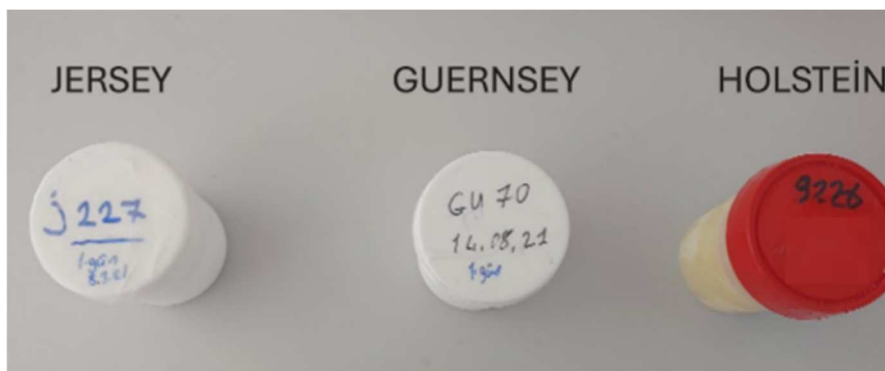


Figure 2. Colostrum samples collected from all three bovine breeds (Holstein, Jersey, and Guernsey) prior to the lactoferrin purification process.

Protein isolation from colostrum was performed using ethanol precipitation. Initially, 10 mL of colostrum from each breed was transferred into falcon tubes and centrifuged at 4°C for 30 minutes at 4000 rpm. When the centrifuge is completed, three distinct phases were observed. The middle phase (supernatant), which contained the target protein (lactoferrin, ~78 kDa), was carefully transferred to a new falcon tube (Figure 3).

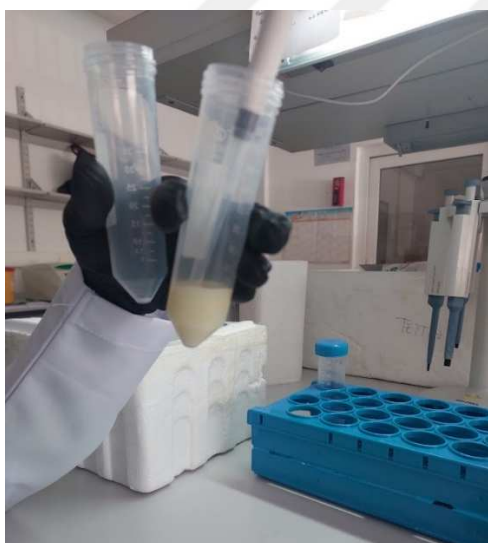


Figure 3. Collection of the middle aqueous phase following centrifugation.

Cold ethanol (1:4, v/v) was added to the supernatant, followed by incubation at -20°C for 1 hour. To purify the proteins and eliminate non-protein impurities, two sequential ethanol incubations were carried out. Following each incubation, the tubes were centrifuged at 4000 rpm for 15 minutes at 4°C, and the resulting supernatants were discarded (Figure 4). The final protein pellet was resuspended in distilled water (1:1, v/v).

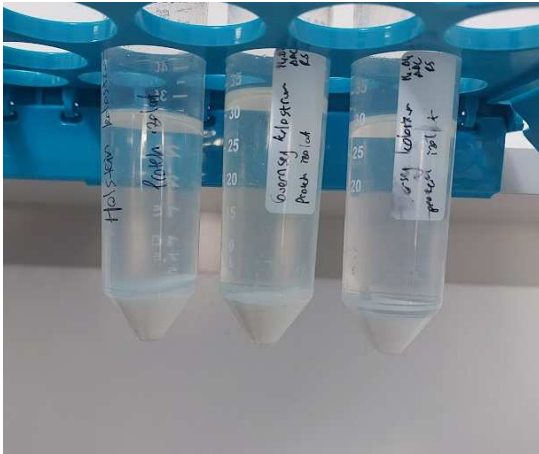


Figure 4. Ethanol precipitation of proteins from the colostrum supernatant.

Determination of Protein Concentration

The concentration of protein isolated from milk samples was quantified using the Qubit™ 3.0 Fluorometer. Following protein isolation, all samples were diluted 1:10 with distilled water. After calibration of the instrument with appropriate standards, 197 μL of Qubit™ Protein Buffer, 1 μL of Qubit™ Protein Reagent, and 2 μL of the sample were added into assay tubes. The tubes were incubated in the dark at room temperature for 15 minutes. Upon completion of incubation, fluorometric measurements were performed. Protein concentrations were determined and then multiplied by the dilution factor. All measurements were performed in duplicate (Figure 5).

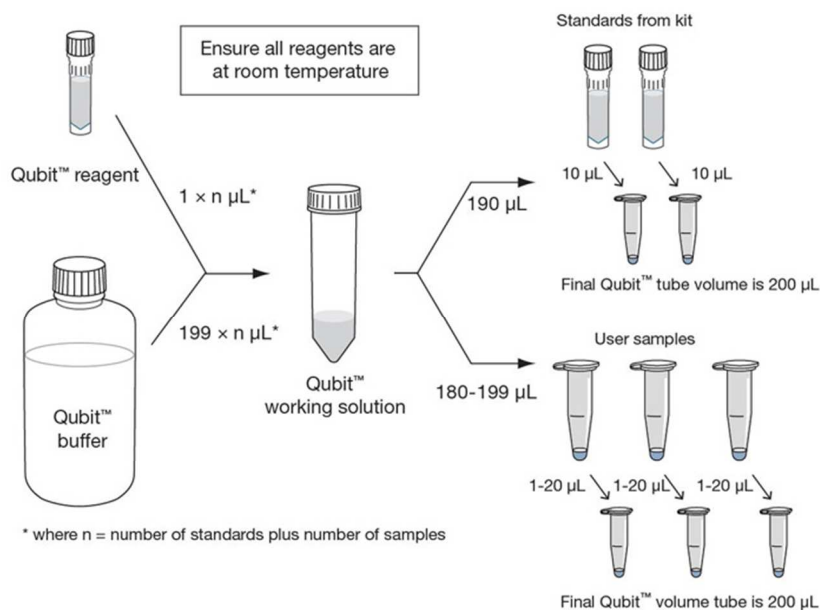


Figure 5. Quantification of protein concentration using the Qubit™ 3.0 Fluorometer Qubit standard/sample preparation workflow (Accessed: 06/14/2025 <https://onelab.andrewalliance.com/library/qubit-fluorometric-quantitation-OY0dx03J>).

Visualization of Isolated Milk Proteins by SDS-PAGE Electrophoresis

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) gel electrophoresis was performed using an Invitrogen Mini Gel Tank to visualize the isolated milk proteins. A 4% stacking gel and a 12% separating gel were prepared for the analysis of protein bands. To estimate the molecular weight of the proteins, the PageRuler™ Plus Prestained Protein Ladder was used. Samples were transferred into 500 µL Eppendorf tubes and mixed with 2× Laemmli Sample Buffer at a 1:1 ratio. The mixture was then denatured by incubation at 95°C for 5 minutes followed by cooling on ice for 2 minutes. Vortexing was performed to ensure homogenization.

After incubation, the gel tank was filled with 1× Tris-Glycine-SDS running buffer. The protein ladder and samples were loaded into the wells and electrophoresed at 200 V for 1 hour. Proteins were then stained with Coomassie Brilliant Blue G-250 dye by shaking at 55 rpm for 30 minutes. Destaining was performed with destaining solution comprising methanol, acetic acid, and distilled water, which was refreshed every 30 minutes until clear visualization of protein bands was achieved. After electrophoresis and Coomassie staining,

the gel was imaged using the iBright™ 750 Imaging System to document the separated protein bands.

3.2.2. Lactoferrin Isolation from Colostrum

The experimental protocol for lactoferrin purification is illustrated in Figure 6.

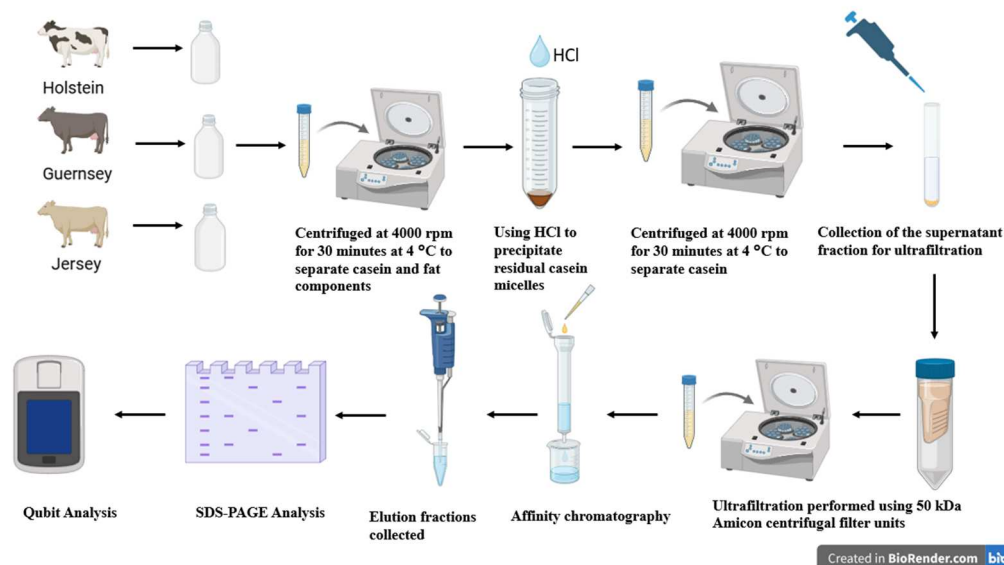


Figure 6. Schematic representation of the experimental workflow for lactoferrin purification from bovine colostrum (*Figure created using BioRender.com.*).

Preliminary Purification and Sample Preparation for Lactoferrin Isolation

Colostrum samples from three different bovine breeds included in the study were individually centrifuged at 4000 rpm for 30 minutes at 4 °C to separate casein and fat components. Following centrifugation, the supernatant layer between the upper fat layer and the lower casein pellet was collected. The pH of the collected supernatant was adjusted to 4.6 using hydrochloric acid (HCl) to precipitate residual casein micelles (Figure 7).



Figure 7. Adjustment of the supernatant pH to 4.6 using HCl for the precipitation of residual casein micelles.

After acid precipitation, samples were re-centrifuged under the same conditions (4000 rpm, 30 minutes, 4 °C). The resulting supernatant was subsequently concentrated via ultrafiltration using Amicon centrifugal filter units with a 50 kDa molecular weight cutoff, centrifuged at 4000 rpm for 270 minutes. At regular intervals during the 270-minute centrifugation, the process was paused and the upper fraction was gently aspirated and dispensed repeatedly using a pipette, in order to prevent clogging of the filter membrane and maintain efficient ultrafiltration (Figure 8).



Figure 8. Ultrafiltration of the supernatant obtained after casein precipitation using Amicon centrifugal filter units with a 50 kDa molecular weight cutoff.

Affinity Chromatography for the Purification of Lactoferrin

Following the preliminary purification steps, the concentrated sample was diluted in a 3:1 ratio with the affinity chromatography running buffer (Table 3) to optimize buffer conditions for chromatographic binding. The resulting mixture was incubated at room temperature (~22–25 °C) on an orbital shaker (55 rpm, 30 minutes) (Figure 9).



Figure 9. Incubation of the diluted sample prior to affinity chromatography.

Affinity chromatography was performed using Heparin-Sepharose beads (Abcam, Cambridge, UK) packed into polypropylene columns. To ensure optimal chromatographic conditions and column equilibration, the column was washed three times with running buffer (Table 3).

Table 3.

Composition of the running buffer used in affinity chromatography (formulated for a final volume of 500 mL).

Component	Amount
Tris-Base (Sigma-Aldrich (T1503))	6.06 g
Tween 20 (Sigma-Aldrich (P9416))	2.50 mL
NaCl (Merck (1.06404))	1.461 g
HCl (Merck)	To adjust pH to 8.0
Ultrapure Water	Up to 500 mL

The concentrated sample obtained from preliminary purification steps was loaded onto the column and passed through twice to maximize binding efficiency and homogenize sample distribution. Subsequently, the sample was reloaded onto the column and incubated at room temperature for three hours to facilitate specific binding between the positively charged lactoferrin and the negatively charged heparin ligand. After incubation, unbound material was washed off, and non-specifically bound proteins were removed by extensive washing with running buffer. The specifically bound proteins were eluted in a stepwise manner using buffers containing increasing concentrations of sodium chloride (NaCl) (0.1 M, 0.25 M, 0.50 M, 0.75 M, and 1 M). These fractions were subjected to SDS-PAGE to evaluate protein content and identify the most prominent band corresponding to lactoferrin. The elution fraction exhibiting the most intense band at the expected molecular weight (~80 kDa) was selected and subsequently concentrated using a 50 kDa molecular weight cutoff Amicon ultrafiltration tube. During ultrafiltration using the 50 kDa Amicon tube, buffer exchange was simultaneously performed to replace the elution buffer with a PNGase F-compatible reaction buffer (Figure 10). This HEPES buffer (Table 4) not only facilitated subsequent enzymatic deglycosylation of lactoferrin but also enabled short-term storage of the sample at +4°C without compromising protein stability.



Figure 10. Buffer exchange using Amicon® Ultra-15 centrifugal filter units with a 50 kDa molecular weight cut-off.

Table 4.

Composition of the HEPES buffer (pH 7.4, Final Volume: 100 mL)

Component	Amount
HEPES (Sigma-Aldrich (H3375))	476 mg
NaCl (Merck (1.06404))	876 mg
HCl (Merck)	To adjust pH to 7.4
Ultrapure Water	Up to 100 mL

3.2.3. N-Glycan Release and Analytical Characterization from Lactoferrin

The next sections describe how N-glycans were released from lactoferrin and also explain how these glycans were analyzed and characterized.

Enzymatic Release of N-glycans From Lactoferrins

Release of N-linked glycans from lactoferrin was carried out using the commercial enzyme PNGase F.

To enzymatically release N-linked glycans, sample tubes were prepared in parallel as experimental (with PNGase F) and control (without enzyme) groups. Each reaction mixture contained glycoprotein, denaturation buffer, GlycoBuffer 2 (10×) and 10% NP-40, and was brought to a final volume of 200 μ L with distilled water. The experimental tubes additionally received 20 μ L of PNGase F, while the control tubes were supplemented with an equal volume of distilled water.

All samples were denatured at 95°C for 10 minutes, rapidly cooled on ice, and subsequently incubated at 37°C for 2 hours to facilitate enzymatic deglycosylation. Eluted glycoprotein fractions from Guernsey, Holstein, and Jersey lactoferrin samples were processed in this manner. As a positive control for enzyme activity, Ribonuclease B (RNase B) was treated with PNGase F under identical buffer conditions and incubated overnight at 37°C. All reaction mixtures were then stored at –20°C for downstream analysis.

Quantitative Determination of Released N-Glycans by Colorimetric Assay

The release of glycans was validated using the Phenol-Sulfuric Acid assay as described before (Masuko et al., 2005). A stock glucose solution was prepared by dissolving 1 mg of glucose in 1 mL of distilled water to achieve a final concentration of 1 mg/mL. Through serial dilutions, glucose standards of 100, 200, 400, 600, 800, and 1000 μ g/mL were prepared, with each dilution adjusted to a total volume of 0.5 mL. An aliquot of 25 μ L from each standard was transferred into the wells of a 96-well microplate.

Subsequently, 25 μ L of 5% (w/v) phenol solution was added to each well, followed by thorough mixing via pipetting. Immediately afterward, 125 μ L of concentrated sulfuric

acid was introduced into each well and rapidly mixed. The lactoferrin samples obtained from the three different bovine breeds were treated identically to the standards, using 25 μ L of sample, 25 μ L of 5% phenol solution, and concentrated sulfuric acid.

All preparations were incubated in the dark at 35 rpm for 30 minutes. After incubation, optical density (OD) values were measured at 490 nm using a microplate reader. Each measurement was performed in duplicate to ensure reproducibility.

Structural Characterization of N-Glycans Using Matrix-Assisted Laser Desorption/Ionization Mass Spectrometry (MALDI-MS)

All samples in which glycan release had been completed and which were subsequently dried were first reconstituted in 50 μ L of water. From each reconstituted sample, 25 μ L was taken and labeled with 2-aminobenzoic acid (2-AA) to enhance MALDI-MS sensitivity. For glycan labeling, stock solutions of 2-AA (48 mg/mL in 30 % acetic acid/70 % Dimethyl sulfoxide (DMSO)) and picoline borane (44.8 mg/mL in 30 % acetic acid/70 % DMSO) were prepared. Aliquots of 12.5 μ L from each stock were mixed in a reaction tube, then combined with the 25 μ L glycan sample and incubated at 65 °C for 2 hours in a Thermomixer. Following labeling, samples were purified using a cotton-based pipette-tip method: cotton was packed into a pipette tip (100 μ L capacity), conditioned by washing five times with water and five times with 85 % acetonitrile (ACN), then the labeled glycans (diluted to 170 μ L with ACN) were loaded by repeated pipetting. The tip was washed five times each with 85 % ACN containing 0.1 % TFA and with 85 % ACN alone. Glycans were eluted by repeatedly aspirating and dispensing 20 μ L of water.

Prior to MALDI-MS analysis, a matrix solution of DHB (5 mg/mL in 50 % ACN, 50 % H₂O, 0.1 % H₃PO₄) was prepared. Mass spectra were acquired on a Rapiflex MALDI-TOF-MS (Bruker Daltonics, Bremen, Germany) equipped with a Smartbeam 3D 10 kHz 355 nm Nd:YAG laser. The instrument was calibrated with an ovalbumin tryptic digest. Data were collected in negative reflectron mode over an m/z range of 1,000–4,000 Da, using 8,000 laser shots at 5,000 Hz. For each sample, 1 μ L of purified glycans and, after drying, 1 μ L of DHB matrix were sequentially spotted onto the MALDI target and allowed to air-dry before analysis.

Detected glycan masses were identified using the Proteinscape glycan search engine (Bruker Daltonics, Bremen-GERMANY) and compared to theoretical glycan mass values to determine the number of glycans present.



CHAPTER FOUR

RESEARCH FINDINGS

4.1. Protein Isolation and Quantification

Proteins were isolated from colostrum samples of different breeds by ethanol precipitation as previously described in detail. The profiles and molecular weights of isolated milk proteins were determined by SDS-PAGE, and the results are presented in Figure 11.

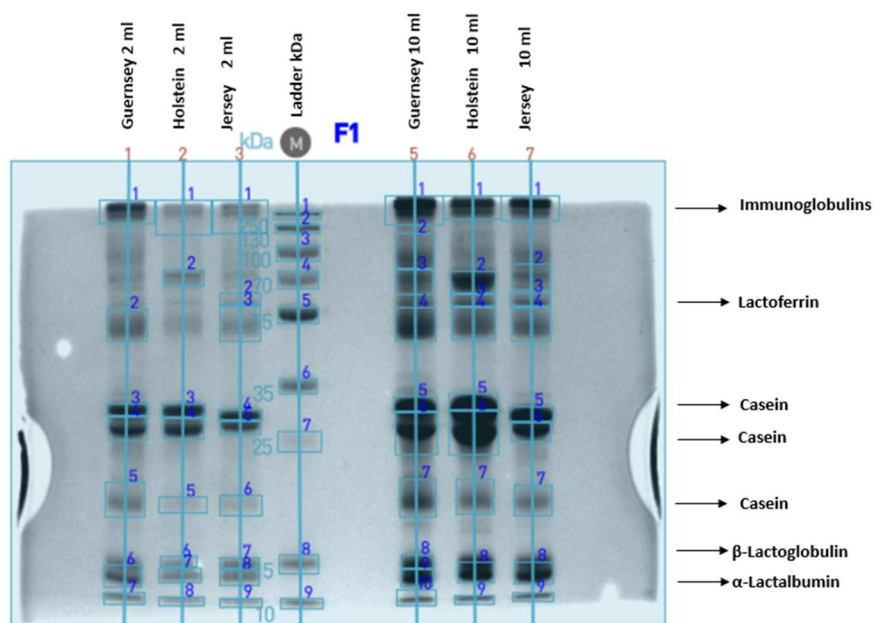


Figure 11. SDS-PAGE profiles of milk proteins isolated from various bovine breeds.

The protein isolates from three bovine breeds were quantified by Qubit fluorometry (Table 5).

Table 5.

Qubit-measured protein concentrations of milk isolates from three bovine breeds.

Breed Name	Protein concentration (mg/mL)	
	First Measurement	Second Measurement
Guernsey	0.962	1.54
Holstein	1.08	1.69
Jersey	1.07	1.49

4.2. Lactoferrin Isolation and Quantification

Lactoferrins specifically bound during affinity chromatography were eluted stepwise using buffers with increasing NaCl concentrations (0.10, 0.25, 0.50, 0.75, and 1.00 M), and each elution fraction was analyzed by SDS-PAGE to identify the NaCl concentration corresponding to the most intense lactoferrin band. It was found that the 0.5 M elution buffer yielded the highest lactoferrin content for all three bovine breeds (The SDS-PAGE results for all three breeds are presented in Appendix 1,2,3.). The 0.5 M elution fractions were quantified by Qubit fluorometry (Table 6).

Table 6.

Qubit-measured lactoferrin concentrations in 0.5 M elution fractions from three bovine breeds.

Breed Name	Protein concentration (mg/mL)	
	First Measurement	Second Measurement
Guernsey	1.16	1.23
Holstein	1.42	1.53
Jersey	1.19	1.27

The 0.5 M elution fractions from all three bovine breeds were subjected to buffer exchange and then analyzed by SDS-PAGE (Figure 12).

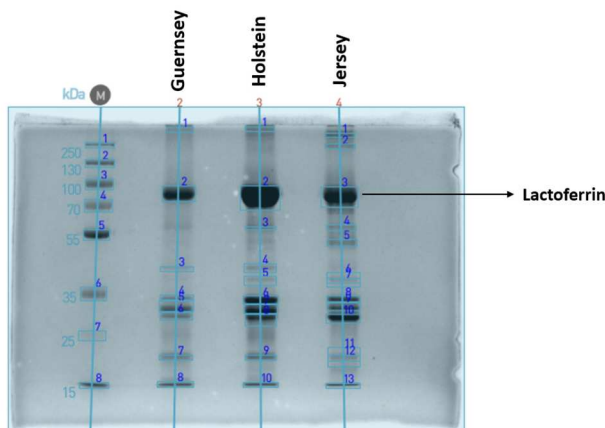


Figure 12. SDS-PAGE analysis of buffer-exchanged 0.5 M elution fractions from three bovine breeds.

4.3. Results of the Colorimetric Quantification of Released N-Glycans

A standard calibration curve was generated using glucose standards (0–1.0 mg/mL) dissolved in the same buffer as the samples. Absorbance at 490 nm was measured for each standard, and the resulting plot exhibited a strong linear relationship ($R^2 = 0.9639$; $y = 0.1398x - 0.001$), confirming assay linearity within this range (Figure 13).

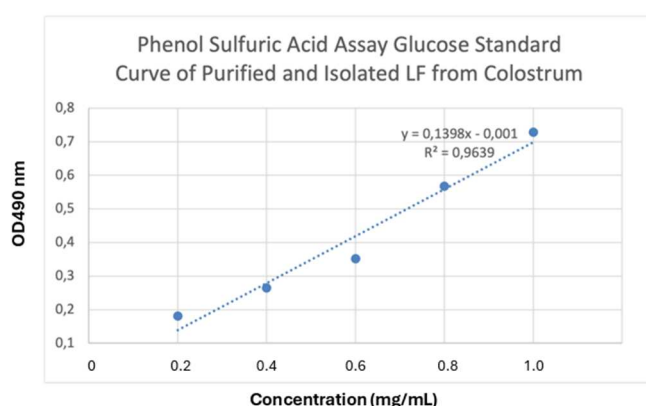


Figure 13. Glucose standard curve (0.2–1.0 mg/mL) for colostrum-derived lactoferrin using the phenol–sulfuric acid assay; linear fit: $y = 0.1398x - 0.001$, $R^2 = 0.9639$.

The phenol–sulfuric acid method was then applied to quantify glycans released from lactoferrin samples before (“Control”) and after PNGase F digestion. Table 7 summarizes the glycan concentrations determined for each bovine breed.

Table 7.

Glycan concentrations measured by phenol–sulfuric acid assay in control and PNGase F–digested lactoferrin samples.

Breed Name	Control ($\mu\text{g/mL}$)	PNGase F Digested ($\mu\text{g/mL}$)	Absolute Difference ($\mu\text{g/mL}$)	Relative Increase (%)
Guernsey	130.6	162.7	32.1	24.7
Holstein	106.7	116.9	10.2	9.6
Jersey	99.1	124.4	25.3	25.6

As shown in Table 7 PNGase F treatment led to a marked increase in detected glycan content for all three breeds. The most pronounced release was observed in Guernsey and Jersey samples, which exhibited ~25 % increases compared to their respective controls, whereas Holstein samples showed a more modest ~10 % increase.

4.4. Characterization of Released N-Glycans from Lactoferrin by MALDI-TOF-MS

Each breed displayed a different N-glycan profile (Appendix 4,5,6). The MALDI-TOF-MS spectra of N-glycans released from lactoferrin samples of different bovine breeds are shown in Figure 14. Guernsey, Holstein, and Jersey lactoferrin contained 38, 44, and 24 distinct N-glycan structures, respectively (Table 8).

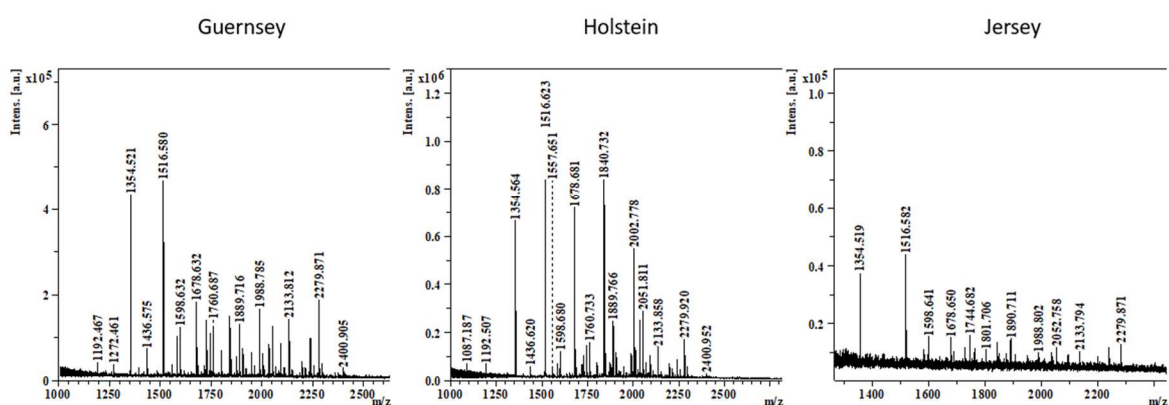


Figure 14. MALDI-TOF-MS spectra of released N-glycans from lactoferrin samples.

Table 8.

N-glycan compositions in Guernsey, Holstein, and Jersey lactoferrin samples, including glycan names and their categories.

	Glycan Composition	Glycan Category	Glycan Category		
			Guernsey	Holstein	Jersey
1	Hex ₃ HexNAc ₂ -AA	Hybrid	+	+	+
2	Hex ₃ HexNAc ₂ Me ₁ -AA	Hybrid	+	+	+

Table 8 continued					
3	Hex4HexNAc2-AA	Hybrid	+	+	
4	Hex5HexNAc2-AA	High-mannose	+	+	+
5	Hex4HexNAc3-AA	Hybrid	+	+	
6	Hex3HexNAc4-AA	Complex bi-antennary (neutral)	+	+	+
7	Hex6HexNAc2-AA	High-mannose	+	+	+
8	Hex5HexNAc3-AA	Hybrid	+	+	
9	Hex3HexNAc4dHex1-AA	Complex Bi- antennary, fucosylated	+	+	+
10	Hex4HexNAc4-AA	Complex bi-antennary (neutral)	+	+	+
11	Hex7HexNAc2-AA	High-mannose	+	+	+
12	Hex4HexNAc3NeuAc1-AA	Complex, sialylated	+	+	+
13	Hex6HexNAc3-AA	Hybrid	+	+	
14	Hex4HexNAc4dHex1-AA	Complex Bi- antennary, fucosylated	+	+	+
15	Hex5HexNAc4-AA	Complex bi-antennary (neutral)	+	+	+
16	Hex4HexNAc5-AA	Complex bi-antennary (neutral)	+	+	+
17	Hex8HexNAc2-AA	High-mannose	+	+	+
18	Hex5HexNAc3NeuAc1-AA	Complex, sialylated	+	+	+
19	Hex7HexNAc3-AA	Hybrid	+	+	
20	Hex4HexNAc4NeuAc1-AA	Complex Bi- antennary, sialylated	+	+	+
21	Hex5HexNAc4dHex1-AA	Complex Bi- antennary, fucosylated	+	+	+
22	Hex6HexNAc4-AA	Complex tri-antennary (neutral)	+		
23	Hex4HexNAc5dHex1-AA	Complex Tri- antennary, fucosylated	+	+	+

Table 8 continued					
24	Hex5HexNAc5-AA	Complex Tri- antennary (neutral)	+	+	
25	Hex9HexNAc2-AA	High-mannose	+	+	
26	Hex6HexNAc3NeuAc1-AA	Complex, sialylated	+	+	
27	Hex4HexNAc4NeuAc1dHex1-AA	Complex Biantennary, sialylated & fucosylated	+	+	+
28	Hex5HexNAc4NeuAc1-AA	Complex Bi- antennary, sialylated	+	+	+
29	Hex7HexNAc4-AA	Complex Tri- antennary (neutral)	+		
30	Hex4HexNAc5NeuAc1-AA	Complex Tri- antennary, sialylated	+	+	+
31	Hex5HexNAc5dHex1-AA	Complex Tri- antennary, fucosylated	+		
32	Hex9HexNAc2dHex1-AA	Complex, fucosylated	+	+	
33	Hex5HexNAc4NeuAc1dHex1-AA	Complex Bi- antennary, sialylated & fucosylated	+	+	+
34	Hex6HexNAc4NeuAc1-AA	Complex Tri- antennary, sialylated	+	+	
35	Hex4HexNAc5NeuAc1dHex1-AA	Complex Tri- antennary, sialylated & fucosylated	+	+	+
36	Hex5HexNAc5NeuAc1-AA	Complex Tri- antennary, sialylated	+	+	
37	Hex6HexNAc4NeuAc1dHex1-AA	Complex Tri- antennary, sialylated & fucosylated	+	+	

Table 8 continued

38	Hex5HexNAc5NeuAc1dHex1-AA	Complex Tri-antennary, sialylated & fucosylated	+	+
39	Hex3HexNAc5dHex1-AA	Complex Tri-antennary & fucosylated		+
40	Hex4HexNAc1-AA	Hybrid	+	+
41	Hex8HexNAc2P1-AA	High-mannose, phosphorylated	+	
42	Hex9HexNAc2P1-AA	High-mannose, phosphorylated	+	
43	Hex6HexNAc5N1-AA	Complex tri-antennary & sialylated (isomer of NeuAc1)	+	
44	Hex5HexNAc4NeuAc2-AA	Complex bi-antennary & di-sialylated	+	
45	Hex6HexNAc5NeuAc1-AA	Complex tri-antennary & sialylated	+	
46	Hex4HexNAc5S1-AA	Complex tri-antennary, sulfated		+
47	Hex3HexNAc2dHex1-AA	Complex, fucosylated	+	
48	Hex4HexNAc3dHex1-AA	Complex biantennary, fucosylated	+	

Analysis of the glycan structures of lactoferrins from all three bovine breeds revealed that complex glycans were the most abundant in all breeds (Figure 15). Holstein exhibited the greatest diversity of complex glycans, whereas Jersey presented lower numbers of complex, hybrid, and high-mannose glycans compared with the other breeds.

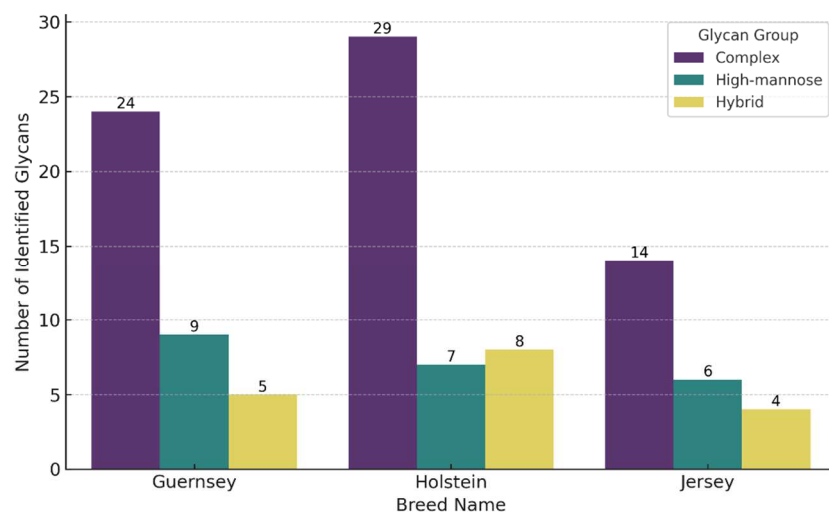


Figure 15. The number of identified glycans in each glycan structural group across the three bovine breeds.

When the Venn diagram was examined (Figure 16), it was observed that the majority of the identified glycans, comprising 22 distinct structures, were common to all three breeds. Regarding breed-specific glycans, Holstein exhibited the highest number with eight unique structures, followed by Guernsey, while Jersey presented only one unique glycan structure. In addition, 13 glycan structures were shared exclusively between Guernsey and Holstein, but were absent in Jersey.

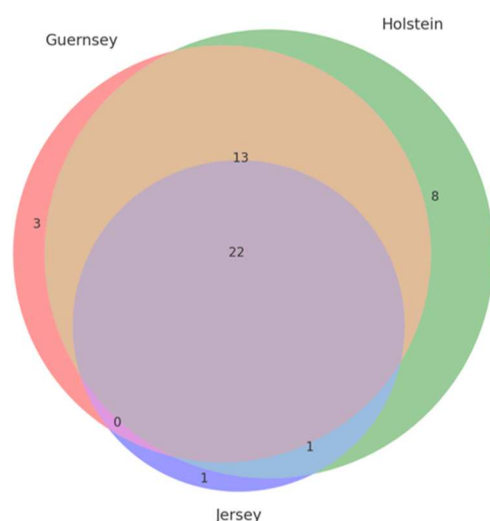


Figure 16. Venn diagram illustrating the distribution of glycan structures among Guernsey, Holstein, and Jersey lactoferrins.

Relative abundance heat map analysis of major N-glycan classes in lactoferrin based on peak areas revealed clear breed-specific patterns (Figure 17). Guernsey and Jersey samples showed predominantly complex glycans (both acidic and neutral), whereas Holstein samples were largely composed of high-mannose structures, with hybrid glycans remaining at low abundance in all three breeds. These results are consistent with previously reported glycosylation patterns of bovine lactoferrin during the colostral stage (Jia et al., 2021). In agreement with previous study, sialic glycans predominated in day one colostrum lactoferrin samples (McJarow and van Amelsfort-Schoonbeek; 2004). Additionally, both fucosylated and sialylated-fucosylated glycans were abundant. It is well established that fucosylated and sialylated N-glycans play an important role in determining the biological activity of bovine lactoferrin (Zlatina and Galuska, 2021). The high levels of sialylation and fucosylation observed in Guernsey and Jersey lactoferrins may contribute to the development of immune-supporting food supplements in the functional dairy industry.

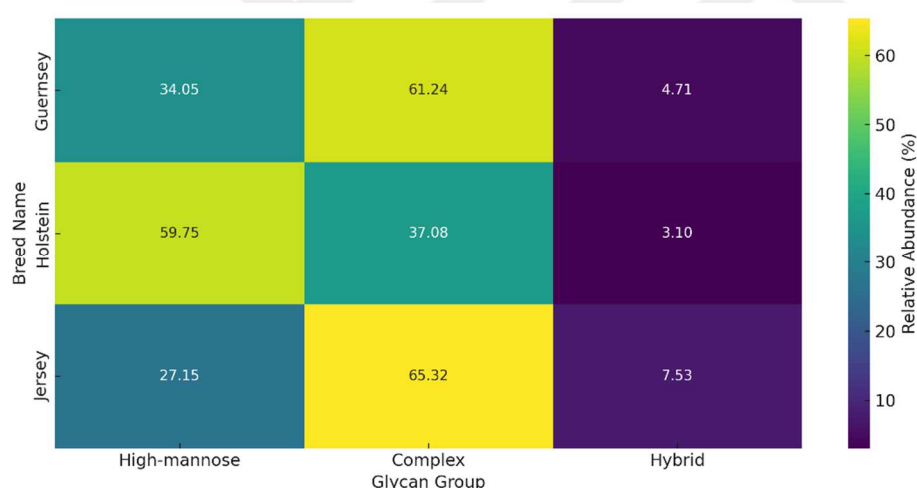


Figure 17. N-glycan class relative abundance among bovine breeds.

CHAPTER FIVE

RESULTS AND RECOMMENDATIONS

This study aimed to investigate the N-glycan profiles of lactoferrin isolated from first-day colostrum samples of Guernsey, Holstein, and Jersey bovine breeds. To the best of our knowledge, this is the first study to comparatively characterize the N-glycan profiles of bovine lactoferrin isolated from first-day colostrum of Guernsey, Holstein, and Jersey breeds using both quantitative and structural analysis. Lactoferrin purification was successfully achieved through ultrafiltration followed by heparin affinity chromatography, and the presence of lactoferrin was confirmed using SDS-PAGE analysis. Subsequently, N-glycans were enzymatically released using PNGase F and quantitatively assessed with the phenol-sulfuric acid method. A noticeable increase in glycan concentration was observed in all samples after enzymatic treatment, with Guernsey and Jersey samples showing greater increases (24.7% and 25.6%, respectively) compared to Holstein (9.6%). These findings suggest that lactoferrins from Guernsey and Jersey may carry a higher number of, or more accessible, N-glycosylation sites, thereby demonstrating breed-dependent differences in N-glycan abundance.

Further structural analysis using MALDI-TOF-MS revealed clear breed-specific differences in glycan diversity and composition. Holstein lactoferrin contained the highest number of distinct glycan structures (44), followed by Guernsey (38) and Jersey (24). In terms of glycan types, Guernsey and Jersey lactoferrins exhibited a higher proportion of complex glycans, whereas Holstein samples had more high-mannose structures. Hybrid glycans were found in relatively low abundance across all breeds, which is likely due to the use of colostrum as the sample source. Hybrid glycans are considered intermediate structures in the biosynthesis pathway of N-glycans, typically occurring during the transition from high-mannose to complex forms. In early lactation, particularly in colostrum, high-mannose glycans are often more dominant due to their role in supporting immune function. Additionally, enzymatic processing in the endoplasmic reticulum and Golgi apparatus may be less active at this stage, limiting the conversion of high-mannose structures into hybrid and complex glycans. First-day colostrum lactoferrins were found to contain high amounts of sialated and fucosylated glycan structures (especially Jersey and Guernsey). This suggests

that first-day colostrum may offer advantages in terms of a wide range of biological activities in future studies.

These results indicate that the extent and patterns of glycosylation in bovine lactoferrin vary by breed. Such differences may impact the biological functions of lactoferrin. Therefore, breed-specific differences in lactoferrin glycan profiles should be taken into account when developing functional dairy products and therapeutic formulations. By integrating multiple analytical techniques such as protein purification, enzymatic deglycosylation, quantitative glycan analysis, and mass spectrometry, this study provides a comprehensive and meaningful contribution to the understanding of breed-specific glycosylation in bovine milk.

Nonetheless, several limitations should be noted. The study was restricted to colostrum samples collected only on the first postpartum day, which reflects early glycosylation patterns and may not represent glycan dynamics throughout lactation. Additionally, the analysis was limited to three breeds from a single source, potentially reducing the generalizability of the results. The structural characterization was performed using MALDI-TOF-MS, which provides information on glycan composition but not on glycosylation sites or linkage types. Future studies should include longitudinal sampling across different lactation stages, a broader range of breeds and geographic sources, and advanced site-specific analyses such as LC-MS/MS. Furthermore, functional assays are recommended to better understand the biological relevance of the observed glycan variations.

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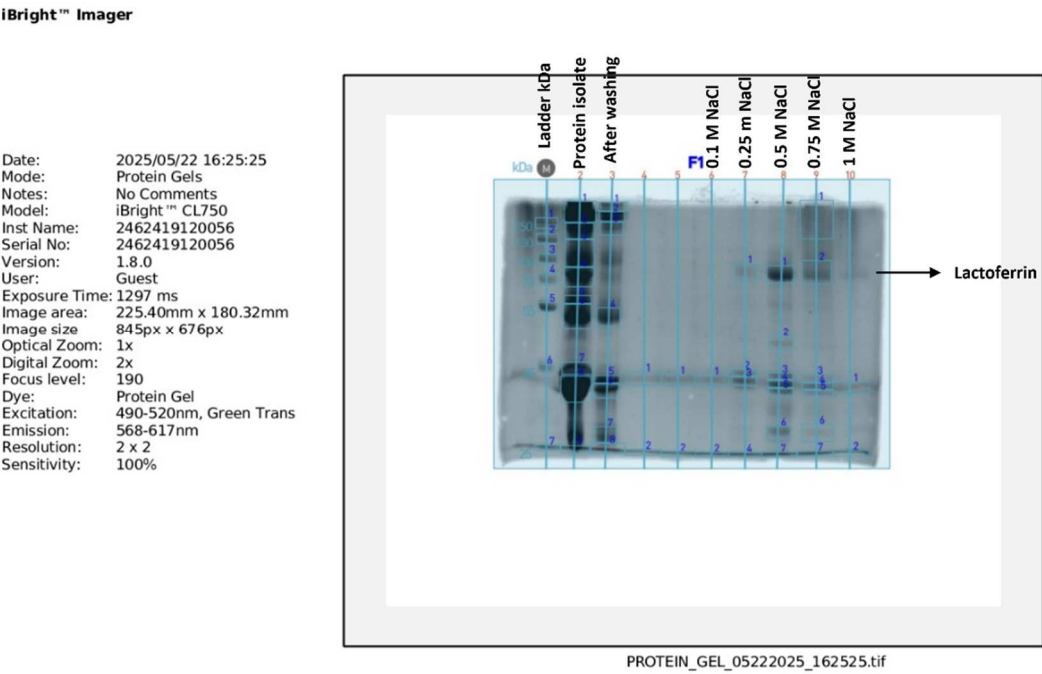
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APPENDICES

APPENDIX 1

SDS-PAGE ANALYSIS OF ELUTION FRACTIONS OF DILUTED LACTOFERRIN ISOLATED FROM GUERNSEY COLOSTRUM



Frame 1 Channel 0**Lane 1**

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	28371200	544	763686	0.150	52152	7.037	6.449
2	25669700	512	1184020	0.203	50136	10.910	5.835
3	22611000	464	1587290	0.270	48730	14.626	5.140
4	24039300	522	1344390	0.340	46052	12.388	5.464
5	22450600	448	3299020	0.438	50113	30.400	5.103
6	12642400	273	976907	0.650	46309	9.002	2.874
7	16947500	370	1696890	0.923	45804	15.636	3.852

Lane 2

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	83963500	1380	19961700	0.110	60843	21.935	14.575
2	60359800	1008	6268040	0.175	59880	6.888	10.478
3	93695600	1599	11163700	0.252	58596	12.267	16.264
4	71959400	1218	9780690	0.335	59080	10.748	12.491
5	30979600	520	3540620	0.412	59576	3.891	5.378
6	86743800	1476	14316000	0.470	58769	15.731	15.058
7	47274000	836	5294730	0.650	56547	5.818	8.206
8	103309999	1716	17313700	0.723	60204	19.025	17.933
9	25493100	504	3365210	0.928	50581	3.698	4.425

Lane 3

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	31331600	644	2851100	0.093	48651	8.337	6.215
2	32871300	555	4673960	0.127	59227	13.668	6.521
3	35051300	660	2177080	0.168	53108	6.366	6.953
4	51409100	945	7978570	0.472	54401	23.331	10.198
5	28047500	494	3260150	0.688	56776	9.533	5.564
6	45332800	836	4320520	0.728	54225	12.634	8.993
7	40696899	792	5512850	0.877	51385	16.121	8.073
8	36587700	784	3422980	0.925	46668	10.009	7.258

Lane 4

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	48267100	1100	1405100	0.685	43879	34.187	11.678
2	24119400	561	2704940	0.940	42993	65.813	5.836

Lane 5

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	35016400	768	1315080	0.688	45594	34.453	8.389
2	19391000	432	2501910	0.945	44886	65.547	4.645

Lane 6

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	37303100	816	1184490	0.692	45714	27.908	8.571
2	19781900	432	3059740	0.947	45791	72.092	4.545

Lane 7

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	42773100	952	1298620	0.320	44929	19.810	9.659
2	20462200	440	560839	0.665	46504	8.556	4.621
3	26720700	540	1684780	0.695	49482	25.701	6.034
4	19866500	423	3011010	0.947	46965	45.933	4.486

Lane 8

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	55578300	1040	8427080	0.325	53440	43.269	12.003
2	35250800	792	1147960	0.562	44508	5.894	7.613
3	19894500	407	931464	0.678	48880	4.783	4.297
4	23627200	444	2152820	0.702	53214	11.054	5.103
5	22985800	468	917551	0.733	49114	4.711	4.964
6	31584400	680	2330790	0.870	46447	11.967	6.821
7	21518300	450	3568330	0.947	47818	18.322	4.647

Lane 9

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	126032000	2640	17175300	0.138	47739	61.007	27.175
2	72928500	1462	5288020	0.317	49882	18.783	15.725
3	29273300	650	183895	0.685	45035	0.653	6.312
4	22414100	460	1311790	0.710	48726	4.660	4.833
5	23811600	516	322636	0.735	46146	1.146	5.134
6	51265900	1196	942430	0.870	42864	3.348	11.054
7	23982600	510	2929010	0.942	47024	10.404	5.171

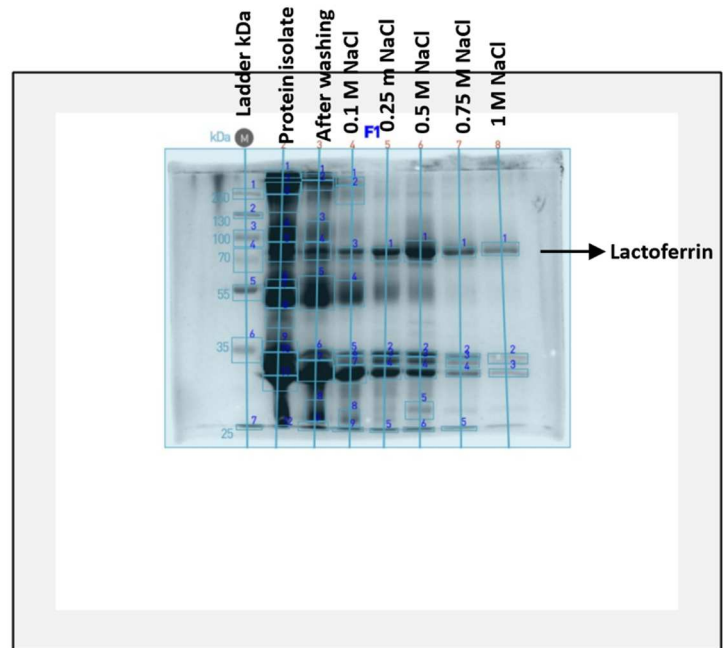
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1	44039700	969	1131250	0.717	45448	37.423	10.130
2	25485800	561	1891640	0.942	45429	62.577	5.862

APPENDIX 2

SDS-PAGE ANALYSIS OF ELUTION FRACTIONS OF DILUTED LACTOFERRIN ISOLATED FROM HOLSTEIN COLOSTRUM

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Lane 1

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	20027100	748	176665	0.150	26774	1.507	5.957
2	13160500	462	1747610	0.221	28485	14.903	3.914
3	29174400	1075	1742870	0.297	27139	14.862	8.678
4	36544000	1470	467735	0.370	24859	3.989	10.870
5	30425000	989	3288460	0.478	30763	28.042	9.049
6	36846100	1505	1394620	0.672	24482	11.893	10.959
7	9445660	333	2908840	0.926	28365	24.805	2.809

Lane 2

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	32142900	650	6751620	0.083	49450	6.213	4.642
2	47169200	850	7014120	0.130	55493	6.454	6.813
3	59308600	1222	10132500	0.179	48534	9.323	8.566
4	51336700	943	10505500	0.282	54439	9.667	7.415
5	62019600	1148	12689000	0.341	54024	11.676	8.958
6	29561700	546	4286830	0.446	54142	3.945	4.270
7	72381500	1305	16620800	0.495	55464	15.294	10.454
8	50782400	1230	4531870	0.564	41286	4.170	7.335
9	38270700	816	6482220	0.659	46900	5.965	5.528
10	88132100	1536	16067000	0.716	57377	14.784	12.729
11	54106700	1104	11801800	0.782	49009	10.860	7.815
12	6503250	154	1793930	0.926	42228	1.651	0.939

Lane 3

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	23557300	506	3228840	0.091	46555	3.829	3.893
2	34726200	736	2546670	0.120	47182	3.020	5.739
3	45843500	1080	6586060	0.272	42447	7.811	7.576
4	47258200	1014	7232090	0.338	46605	8.577	7.810
5	119971000	2450	29970000	0.478	48967	35.543	19.826
6	38988600	882	5139740	0.684	44204	6.096	6.443
7	81107400	1450	14544000	0.740	55936	17.248	13.404
8	42473500	930	11356700	0.873	45670	13.469	7.019
9	20926700	588	3716050	0.924	35589	4.407	3.458

Lane 4

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	16939300	520	1339630	0.105	32575	2.777	3.496
2	36156700	1148	2016050	0.152	31495	4.179	7.462
3	32472900	800	6598300	0.348	40591	13.678	6.701
4	70762800	1634	15365700	0.480	43306	31.852	14.603
5	15172100	396	2402040	0.681	38313	4.979	3.131
6	21128800	520	712077	0.706	40632	1.476	4.360
7	58315900	1196	14013000	0.750	48759	29.048	12.035
8	26402800	775	3297340	0.897	34068	6.835	5.449

9	11429500	352	2496750	0.934	32470	5.176	2.359
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Lane 5

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	37761400	860	10102500	0.348	43908	34.514	8.618
2	16252100	378	3829900	0.681	42995	13.085	3.709
3	25016400	574	2444000	0.706	43582	8.350	5.709
4	40792600	900	10354600	0.745	45325	35.375	9.309
5	9977010	320	2539620	0.936	31178	8.676	2.277

Lane 6

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	67108600	1426	21238800	0.341	47060	47.962	14.845
2	17281600	405	4158280	0.684	42670	9.390	3.823
3	24870300	588	2338230	0.708	42296	5.280	5.502
4	33884500	765	9867110	0.748	44293	22.282	7.496
5	28401500	1040	3838120	0.870	27309	8.667	6.283
6	10676000	352	2842440	0.934	30329	6.419	2.362

Lane 7

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	29327600	768	7248000	0.341	38186	40.614	8.338
2	12257100	392	2065170	0.689	31268	11.572	3.485
3	18852700	611	1592140	0.713	30855	8.921	5.360
4	18941400	588	4478570	0.748	32213	25.095	5.385
5	11119400	450	2462270	0.931	24709	13.797	3.161

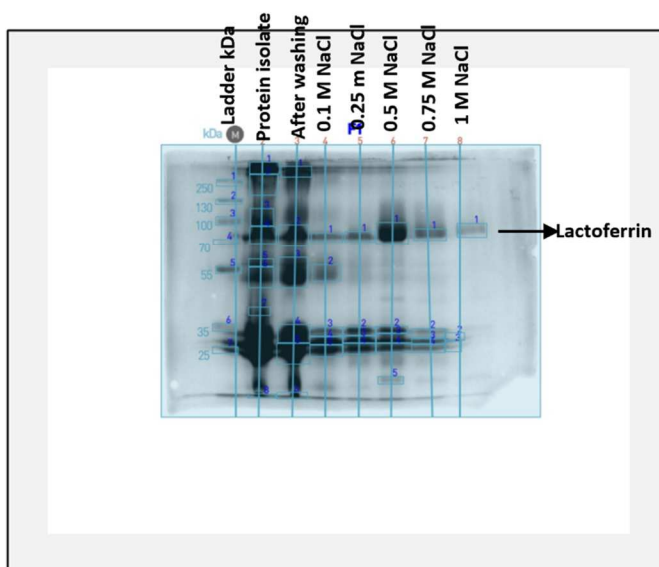
Lane 8

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1	31641200	1040	6443780	0.331	30424	57.277	9.909
2	20541700	840	2397530	0.699	24454	21.311	6.433
3	19025600	770	2408850	0.745	24708	21.412	5.958

APPENDIX 3

SDS-PAGE ANALYSIS OF ELUTION FRACTIONS OF DILUTED LACTOFERRIN ISOLATED FROM JERSEY COLOSTRUM

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Sensitivity: 100%



PROTEIN_GEL_05202025_202406.tif

Lane 1

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	12964300	440	884640	0.139	29464	7.414	3.319
2	12326400	410	1493720	0.207	30064	12.518	3.155
3	17993800	560	2105990	0.277	32131	17.650	4.606
4	10495300	370	453596	0.355	28365	3.801	2.687
5	19266300	520	3203130	0.461	37050	26.845	4.932
6	15222199	494	1052720	0.668	30814	8.823	3.897
7	17660300	468	2738280	0.751	37735	22.949	4.521

Lane 2

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	49176900	874	13371400	0.086	56266	22.715	8.163
2	66675400	1408	6922370	0.146	47354	11.759	11.067
3	51169600	972	8408390	0.264	52643	14.284	8.494
4	58695600	1107	10257000	0.330	53022	17.424	9.743
5	24840800	494	3009130	0.433	50285	5.112	4.123
6	60915300	1216	9712440	0.486	50094	16.499	10.111
7	24761900	510	3330060	0.610	48552	5.657	4.110
8	14657400	387	3855550	0.919	37874	6.550	2.433

Lane 3

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	41976000	782	6837120	0.098	53677	7.825	7.322
2	74367100	1530	15333400	0.330	48605	17.548	12.972
3	101499999	1978	29035300	0.469	51314	33.230	17.705
4	76990800	1421	15334800	0.690	54180	17.550	13.430
5	86014199	1600	15807700	0.763	53758	18.091	15.004
6	20558700	552	5029160	0.917	37244	5.756	3.586

Lane 4

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	28360500	752	4874290	0.338	37713	19.299	6.911
2	44027200	1118	7578740	0.469	39380	30.007	10.729
3	29241799	735	2287990	0.683	39784	9.059	7.126
4	33109000	637	3736680	0.715	51976	14.795	8.068
5	48770700	980	6778590	0.753	49766	26.839	11.884

Lane 5

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	31297000	828	5619340	0.335	37798	27.999	8.380
2	30191700	686	5808770	0.680	44011	28.943	8.084
3	29719900	588	3260340	0.710	50544	16.245	7.957
4	42644800	882	5381040	0.746	48350	26.812	11.418

Lane 6

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	79193300	1617	27566500	0.322	48975	62.103	19.574
2	25227000	576	6075560	0.675	43796	13.687	6.235
3	38099100	795	4048850	0.708	47923	9.121	9.417
4	38407500	848	4261620	0.743	45291	9.601	9.493
5	14187600	520	2435800	0.861	27283	5.487	3.507

Lane 7

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	35041300	950	8121700	0.327	36885	56.751	11.252
2	25436200	816	844916	0.688	31171	5.904	8.168
3	18718800	459	3063700	0.718	40781	21.408	6.011
4	25818700	714	2280740	0.743	36160	15.937	8.291

Lane 8

Band ID	Volume	Area	Local Bg. Corr. Vol.	Rf	Density	Band Purity %	Lane Purity %
1	28316700	1058	4901540	0.312	26764	78.768	11.444
2	9368230	377	495821	0.703	24849	7.968	3.786
3	10493500	408	825365	0.738	25719	13.264	4.241

APPENDIX 4

IDENTIFICATION OF N-GLYCAN STRUCTURES OF GUERNSEY LACTOFERRIN

Row	Composition	Accession	m/z meas.	z	m/z calc.	Δ MH+ [Da]	Int.	Area
1	Hex3HexNac2-AA	5214	1031,1843	-1	1031,3819	-0,1977	22912	2286
2	Hex3HexNac2Me1-AA	8257	1045,1770	-1	1045,3976	-0,2206	21876	2350
3	Hex4HexNac2-AA	5735	1192,4670	-1	1192,4261	0,0409	38549	7521
4	Hex5HexNac2-AA	4411	1354,5214	-1	1354,4789	0,0425	397712	87573
5	Hex4HexNac3-AA	4555	1395,5376	-1	1395,5055	0,0321	23409	7038
6	Hex3HexNac4-AA	1028	1436,5747	-1	1436,5320	0,0427	59197	13826
7	Hex6HexNac2-AA	4412	1516,5804	-1	1516,5317	0,0487	423485	113189
8	Hex5HexNac3-AA	8264	1557,6003	-1	1557,5583	0,0420	31501	9638
9	Hex3HexNac4dHex1-AA	4550	1582,6392	-1	1582,5899	0,0493	95900	28185
10	Hex4HexNac4-AA	4554	1598,6322	-1	1598,5848	0,0473	111982	34418
11	Hex7HexNac2-AA	5147	1678,6318	-1	1678,5846	0,0472	163111	54660
12	Hex4HexNac3NeuAc1-AA	4553	1686,6481	-1	1686,6009	0,0473	26070	9128
13	Hex6HexNac3-AA	1710	1719,6428	-1	1719,6111	0,0316	26008	8711
14	Hex4HexNac4dHex1-AA	5385	1744,6886	-1	1744,6427	0,0458	90080	31809
15	Hex5HexNac4-AA	4894	1760,6870	-1	1760,6377	0,0493	104032	37167
16	Hex4HexNac5-AA	8187	1801,7069	-1	1801,6642	0,0427	62650	22603
17	Hex8HexNac2-AA	5148	1841,7059	-1	1841,6460	0,0599	112868	50116
18	Hex5HexNac3NeuAc1-AA	209	1848,6870	-1	1848,6537	0,0333	44573	17993
19	Hex7HexNac3-AA	6814	1881,6509	-1	1881,6639	-0,0130	20952	8534
20	Hex4HexNac4NeuAc1-AA	6949	1889,7159	-1	1889,6803	0,0356	112687	48534
21	Hex5HexNac4dHex1-AA	5883	1906,7288	-1	1906,6956	0,0332	61245	24008
22	Hex6HexNac4-AA	4534	1922,6766	-1	1922,6905	-0,0139	21390	11596
23	Hex4HexNac5dHex1-AA	4551	1947,7611	-1	1947,7221	0,0390	60377	23088
24	Hex5HexNac5-AA	6815	1963,7509	-1	1963,7170	0,0339	28288	10781
25	Hex9HexNac2-AA	6654	2002,7246	-1	2002,6902	0,0344	61138	26813
26	Hex6HexNac3NeuAc1-AA	7569	2010,7257	-1	2010,7065	0,0191	25319	14363
27	Hex4HexNac4NeuAc1dHex1-AA	4687	2035,7620	-1	2035,7382	0,0239	78261	35702
28	Hex5HexNac4NeuAc1-AA	4556	2051,7591	-1	2051,7331	0,0261	117346	55707
29	Hex7HexNac4-AA	8984	2084,7420	-1	2084,7433	-0,0013	17195	9067
30	Hex4HexNac5NeuAc1-AA	8119	2092,7851	-1	2092,7596	0,0255	75280	34613
31	Hex5HexNac5dHex1-AA	5782	2109,7897	-1	2109,7749	0,0148	26559	11962
32	Hex9HexNac2dHex1-AA	16210	2149,8000	-1	2149,7568	0,0432	22496	8299
33	Hex5HexNac4NeuAc1dHex1-AA	8136	2197,8127	-1	2197,7910	0,0217	41111	18397
34	Hex6HexNac4NeuAc1-AA	7924	2213,8028	-1	2213,7859	0,0169	28235	12264
35	Hex4HexNac5NeuAc1dHex1-AA	8116	2238,8458	-1	2238,8175	0,0283	93952	45227
36	Hex5HexNac5NeuAc1-AA	16255	2254,8312	-1	2254,8124	0,0188	32613	15858
37	Hex6HexNac4NeuAc1dHex1-AA	7925	2359,8630	-1	2359,8438	0,0192	13735	7633
38	Hex5HexNac5NeuAc1dHex1-AA	4685	2400,9052	-1	2400,8704	0,0348	28072	15312

APPENDIX 5

IDENTIFICATION OF N-GLYCAN STRUCTURES OF HOLSTEIN LACTOFERRIN

Row	Composition	Accession	m/z meas.	z	m/z calc.	Δ MH+ [Da]	Int.	Area
1	Hex4HexNac1-AA	5745	989,2079	-1	989,3467	-0,1388	88213	16127
2	Hex3HexNac2-AA	5214	1030,4766	-1	1030,3733	0,1033	85839	10148
3	Hex3HexNac2-AA	5214	1031,1922	-1	1031,3819	-0,1898	66111	9128
4	Hex3HexNac2Me1-AA	8257	1045,1925	-1	1045,3976	-0,2051	64537	9182
5	Hex3HexNac2dHex1-AA	1020	1177,1790	-1	1177,4398	-0,2608	49131	7821
6	Hex4HexNac2-AA	5735	1192,5074	-1	1192,4261	0,0813	232745	30813
7	Hex5HexNac2-AA	4411	1354,5614	-1	1354,4789	0,0825	2034474	437903
8	Hex4HexNac3-AA	4555	1395,5860	-1	1395,5055	0,0806	65831	13526
9	Hex3HexNac4-AA	1028	1436,6187	-1	1436,5320	0,0866	109496	26267
10	Hex6HexNac2-AA	4412	1516,6171	-1	1516,5317	0,0854	2895184	924492
11	Hex4HexNac3dHex1-AA	4566	1541,6458	-1	1541,5634	0,0824	49003	12394
12	Hex5HexNac3-AA	8264	1557,6495	-1	1557,5583	0,0912	104486	30034
13	Hex3HexNac4dHex1-AA	4550	1582,6826	-1	1582,5899	0,0927	163673	50520
14	Hex4HexNac4-AA	4554	1598,6777	-1	1598,5848	0,0928	279022	92301
15	Hex7HexNac2-AA	5147	1678,6781	-1	1678,5846	0,0936	2110365	815602
16	Hex4HexNac3NeuAc1-AA	4553	1686,6822	-1	1686,6009	0,0813	103805	34691
17	Hex6HexNac3-AA	1710	1719,7031	-1	1719,6111	0,0920	141497	54473
18	Hex4HexNac4dHex1-AA	5385	1744,7350	-1	1744,6427	0,0922	299332	119412
19	Hex5HexNac4-AA	4894	1760,7310	-1	1760,6377	0,0934	339830	141566
20	Hex3HexNac5dHex1-AA	8049	1785,7546	-1	1785,6693	0,0853	37126	11906
21	Hex4HexNac5-AA	8187	1801,7532	-1	1801,6642	0,0890	136911	56967
22	Hex8HexNac2-AA	5148	1840,7231	-1	1840,6374	0,0858	2240737	1151826
23	Hex5HexNac3NeuAc1-AA	209	1848,7314	-1	1848,6537	0,0777	248283	112388
24	Hex7HexNac3-AA	6814	1881,7406	-1	1881,6639	0,0767	98089	44989
25	Hex4HexNac4NeuAc1-AA	6949	1889,7563	-1	1889,6803	0,0761	579742	287128
26	Hex5HexNac4dHex1-AA	5883	1907,7963	-1	1907,7042	0,0921	63544	21266
27	Hex8HexNac2P1-AA	10168	1921,7343	-1	1921,6124	0,1219	59930	24999
28	Hex4HexNac5dHex1-AA	4551	1947,8034	-1	1947,7221	0,0813	103203	47198
29	Hex5HexNac5-AA	6815	1963,7854	-1	1963,7170	0,0683	46298	16503
30	Hex9HexNac2-AA	6654	2002,7689	-1	2002,6902	0,0786	1510976	835204
31	Hex6HexNac3NeuAc1-AA	7569	2010,7713	-1	2010,7065	0,0648	321201	172110
32	Hex4HexNac4NeuAc1dHex1-AA	4687	2035,8041	-1	2035,7382	0,0660	629484	345808
33	Hex5HexNac4NeuAc1-AA	4556	2051,7973	-1	2051,7331	0,0642	768985	438222
34	Hex9HexNac2P1-AA	8555	2082,7695	-1	2082,6565	0,1129	43753	17820
35	Hex4HexNac5NeuAc1-AA	8119	2092,8217	-1	2092,7596	0,0621	251281	140397
36	Hex6HexNac5N1-AA	1891	2108,8265	-1	2108,7680	0,0585	80246	39499
37	Hex9HexNac2dHex1-AA	16210	2149,8360	-1	2149,7568	0,0792	76427	39411
38	Hex5HexNac4NeuAc1dHex1-AA	8136	2197,8484	-1	2197,7910	0,0574	165786	98966
39	Hex6HexNac4NeuAc1-AA	7924	2213,8409	-1	2213,7859	0,0550	100938	57586
40	Hex4HexNac5NeuAc1dHex1-AA	8116	2238,8686	-1	2238,8175	0,0510	189116	118076
41	Hex5HexNac5NeuAc1-AA	16255	2254,8643	-1	2254,8124	0,0518	88257	49008
42	Hex5HexNac4NeuAc2-AA	4706	2343,7662	-1	2343,8372	-0,0710	21923	14678
43	Hex6HexNac4NeuAc1dHex1-AA	7925	2359,8635	-1	2359,8438	0,0197	27858	16671
44	Hex5HexNac5NeuAc1dHex1-AA	4685	2400,9255	-1	2400,8704	0,0551	52385	32646
45	Hex6HexNac5NeuAc1-AA	16722	2416,9112	-1	2416,8653	0,0460	27996	14457

APPENDIX 6

IDENTIFICATION OF N-GLYCAN STRUCTURES OF JERSEY LACTOFERRIN

Row	Composition	Accession	m/z meas.	z	m/z calc.	Δ MH+ [Da]	Int.	Area
1	Hex4HexNAc1-AA	5745	989,1974	-1	989,3467	-0,1493	78049	14390
2	Hex3HexNAc2-AA	5214	1031,1888	-1	1031,3819	-0,1931	61663	8504
3	Hex3HexNAc2Me1-AA	8257	1045,1791	-1	1045,3976	-0,2185	59759	7734
4	Hex5HexNAc2-AA	4411	1354,5505	-1	1354,4789	0,0716	176074	37294
5	Hex3HexNAc4-AA	1028	1436,6048	-1	1436,5320	0,0727	44064	8404
6	Hex6HexNAc2-AA	6296	1516,6149	-1	1516,5317	0,0831	181028	46319
7	Hex3HexNAc4dHex1-AA	4550	1582,6700	-1	1582,5899	0,0800	52442	12047
8	Hex4HexNAc4-AA	4554	1598,6664	-1	1598,5848	0,0815	58286	16100
9	Hex7HexNAc2-AA	5147	1678,6670	-1	1678,5846	0,0824	57085	14779
10	Hex4HexNAc3NeuAc1-AA	4553	1686,6574	-1	1686,6009	0,0565	35279	8303
11	Hex4HexNAc4dHex1-AA	5385	1744,7262	-1	1744,6427	0,0834	65600	19988
12	Hex5HexNAc4-AA	4894	1760,7101	-1	1760,6377	0,0725	53947	17926
13	Hex4HexNAc5-AA	8187	1801,7261	-1	1801,6642	0,0619	39779	12341
14	Hex8HexNAc2-AA	5148	1840,6857	-1	1840,6374	0,0483	40930	12111
15	Hex5HexNAc3NeuAc1-AA	209	1848,6975	-1	1848,6537	0,0438	36004	14637
16	Hex4HexNAc5S1-AA	10516	1881,5794	-1	1881,6210	-0,0417	34045	13471
17	Hex4HexNAc4NeuAc1-AA	6949	1889,7338	-1	1889,6803	0,0535	62041	21828
18	Hex5HexNAc4dHex1-AA	5883	1906,7586	-1	1906,6956	0,0630	51249	19389
19	Hex4HexNAc5dHex1-AA	4551	1947,7814	-1	1947,7221	0,0593	35761	13785
20	Hex4HexNAc4NeuAc1dHex1-AA	4687	2035,7794	-1	2035,7382	0,0413	43310	16700
21	Hex5HexNAc4NeuAc1-AA	4556	2051,7765	-1	2051,7331	0,0435	59320	22532
22	Hex4HexNAc5NeuAc1-AA	8119	2092,8030	-1	2092,7596	0,0434	45980	17091
23	Hex5HexNAc4NeuAc1dHex1-AA	8136	2197,8262	-1	2197,7910	0,0352	33235	13527
24	Hex4HexNAc5NeuAc1dHex1-AA	2973	2238,8539	-1	2238,8175	0,0363	39078	17824

APPENDIX 7

ORAL PRESENTATION WITHIN THE SCOPE OF THIS THESIS

12th International Artemis Scientific Research Congress II
Congress Proceedings Book

PURIFICATION AND QUANTITATIVE COMPARISON OF LACTOFERRIN FROM COLOSTRUM OF DIFFERENT CATTLE BREEDS

Rüya SESSİZ^{1,2}, Sercan KARAV³

¹ Çanakkale Onsekiz Mart University, Faculty of Dentistry, Department of Dentomaxillofacial Radiology, Çanakkale, TÜRKİYE

² Çanakkale Onsekiz Mart University, School of Graduate Studies, Department of Molecular Biology and Genetics, Çanakkale, TÜRKİYE

³ Çanakkale Onsekiz Mart University, Faculty of Science, Department of Molecular Biology and Genetics, Çanakkale, TÜRKİYE

ABSTRACT

Colostrum is a specialized form of milk secreted during the initial days postpartum, characterized by its rich content of bioactive components that support the immune system. Among these components, lactoferrin is a glycoprotein notable for its antimicrobial, antiviral, antioxidant, and immunomodulatory properties. The concentration of lactoferrin may vary depending on the stage of lactation, the breed of the animal, and environmental conditions.

In this study, lactoferrin was purified from colostrum samples obtained from Jersey, Holstein, and Guernsey breeds, and their quantities were compared. The samples were initially subjected to centrifugation to separate fat, casein, and cellular components, and the middle-phase supernatant was collected. The isolated fraction was then treated with HCl precipitation to remove residual casein micelles, followed by a second centrifugation step. The resulting supernatant was concentrated approximately 20-fold using Amicon ultrafiltration tubes with a molecular weight cutoff of 50 kDa. The concentrated samples were subjected to affinity chromatography using a heparin Sepharose column, and elution was performed with five different NaCl (0.1 M-1 M) concentrations. The collected fractions were analyzed by SDS-PAGE, and lactoferrin-specific bands were identified. The most intense fractions were subjected to buffer exchange to reduce impurities and then re-quantified using the Qubit fluorometric system, while protein purity was assessed by SDS-PAGE.

According to the results, the average lactoferrin concentrations were 1.23 mg/mL in Guernsey, 1.27 mg/mL in Jersey, and 1.53 mg/mL in Holstein samples. These findings suggest that the biofunctional content of colostrum varies by cattle breed, highlighting an important parameter to consider in the production of bio functional dairy products.

Keywords: Affinity chromatography; colostrum; lactoferrin