T.C.

ÇANAKKALE ONSEKIZ MART UNIVERSITY

SCHOOL OF GRADUATE STUDIES

DEPARTMENT OF MOLECULAR BIOLOGY AND GENETICS

DETERMINATION OF BIOACTIVE MILK *N*-GLYCANS IN DIFFERENT CATTLE BREEDS

MASTER OF SCIENCE THESIS

EDA NTELITZE

Thesis supervisor:

Assoc. Prof. Dr. SERCAN KARAV

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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 that I prepared following the Thesis Writing Rules of School of Graduate Studies of Çanakkale Onsekiz Mart University; I declare that I have obtained the data, information, and documents I presented in the thesis within the framework of academic and ethical rules, I have presented all information, documents, evaluations, and results following scientific ethics and ethical rules, I cited all the works that I used in my thesis study by making appropriate reference, I did not make any changes in the data used and that the study I presented in this thesis is original. Otherwise, I undertake and declare that I accept all loss of rights that may arise against me.

(İmza)
Eda NTELITZE
17/08/2022

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Eda NTELITZE

Canakkale, August 2022

ÖZET

ÇEŞİTLİ SIĞIR IRKLARININ BİYOAKTİF SÜT *N*-GLİKANLARININ BELİRLENMESİ

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Moleküler Biyoloji ve Genetik Anabilim Dalı Yüksek Lisans Tezi
Danışman: Doç. Dr. Sercan KARAV
17/08/2022, 57

Yaşamın ilk anından itibaren besin ve enerji kaynağı rolünü üstlenmiş ve hayvansal kökenli gıdaların başında gelen sütün, içermiş olduğu protein ve glikanlarla birlikte prebiyotik ve antimikrobiyal etki de gösterdiği belirtilmiştir. Süt gelişme çağındaki tüketiciler için büyümeyi arttırması ve büyüme faktörlerini teşvik etmesi için gereken tüm mikro elementleri karşılar. Hatta patojen enfeksiyonlarının oluşumunu azaltmaya yardımcı olup, bağırsak epitelyumunun gelişmesini teşvik ederek bebekleri koruma özelliği rolünü üstlendiği de bilinmektedir. Süt biyoaktif peptitlerin ana kaynağı olarak görülür. Canlıların temel besin kaynağı olan sütün besin değerini oluşturan protein içeriği oldukça önemlidir. Süt proteinleri iki kategori altında incelenmekte olup, bunlar kazein ve whey proteinidir. Sütün antimikrobiyal özelliği whey proteinlerinden sağlanır ve çoğunu immünoglobulin G (IgG) ve laktoferrin oluşturmaktadır. Sütteki *N*-glikanların başlıca kaynağını IgG ve laktoferrin oluşturur.

Bu çalışmada, farklı ırklara ait sütlerin protein konsantrasyonları belirlenmiş ve protein profilleri görüntülenmiştir. Sonrasında farklı ırklara ait süt proteinlerinin içermiş olduğu *N*-glikanların salınımı, PNGaz F olarak da bilinen peptidil *N*-glikosidaz F enzimi kullanılarak sağlanmıştır. Elde edilen serbest glikanların konsantrasyonları Fenol Sülfürik asit yöntemi ile belirlenmiştir. Böylece saflaştırılan glikanların yapıları MALDI-MS analizleri ile karakterize edilmiştir. Son olarak *N*-glikanların ana kaynağını oluşturan ve ayrıca antimikrobiyal özellik gösteren IgG ve antioksidant özelliğe sahip laktoferrin konsantrayonları belirlenerek, tüketiciler için sağlık açısından hangi ırkın sütünün besin

kaynağı bakımından zengin ve faydalı olabileceği sunulmuştur. Ayrıca hayvancılık sektörü ile ilgilenen birimler için de yüksek proteinli süt üreten ırklar ortaya konulmuştur.

Anahtar Kelimeler: Süt, Protein, *N*-glikan, İmmünoglobulin G (IgG), Laktoferrin

ABSTRACT

DETERMINATION OF BIOACTIVE MILK *N*-GLYCANS IN DIFFERENT CATTLE BREEDS

Eda NTELITZE

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School of Graduate Studies

Master of Science Thesis in Molecular Biology and Genetics

Advisor: Assoc. Prof. Dr. Sercan KARAV

17/08/2022, 57

It was stated that milk, which has the role of nutrient and energy source from the beginning of life, being one of the animal originated foods, also shows prebiotic and antimicrobial effects together with the proteins and glycans it contains. Milk provides all the microelements needed to increase the growth and stimulate growth factors for consumers at developmental age. It is even known that it helps to reduce the formation of pathogen infections and plays the role of protecting babies by giving countenance to gut epithelium growth. Milk is seen main source of bioactive peptides. Protein content forming the milk's feeding worth, the major source for beings, is significant. Milk proteins categorized in casein and whey protein. The antimicrobial property is provided by whey proteins, mostly from immunoglobulin G (IgG) and lactoferrin. IgG and lactoferrin are main *N*-glycan sources in milk.

In this study, protein concentrations of different breeds' milk were determined and protein profiles monitored. Afterwards, from milk proteins the *N*-glycan release achieved by using peptidyl *N*-glycosidase F enzyme, known as PNGase F. The concentrations of free glycans were determined by Phenol Sulfuric acid method. Thus, structures of purified glycans were characterized by MALDI-MS analysis. Finally, the concentrations of IgG, being the main source of *N*-glycans and exhibiting antimicrobial properties, and lactoferrin with antioxidant properties were determined, was presented which breed's milk could be rich and beneficial regarding the consumers' health. Furthermore, protein-rich milk producing breeds were presented for husbandry sector.

Keywords: Milk, Protein, *N*-glycan, Immunoglobulin G (IgG), Lactoferrin

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SYMBOLS AND ABBREVIATIONS

LF	Lactoferrin
IgG	Immunoglobulin G
HRP	Horseradish Peroxide
HMO	Human Milk Oligosaccharide
BMO	Bovine Milk Oligosaccharide
ELISA	Enzyme-linked Immunoassay
LPO	Lactoperoxidase
Lys	Lysozyme
ER	Endoplasmic Reticulum
N-glycan	*N*-linked glycan
PNGase F	Peptidyl *N*-glycosidase F
DNA	Deoxyribonucleic Acid
RNA	Ribonucleic Acid
Thr	Threonine
Ser	Serine
Asn	Asparagine
dH_2O	Distilled water
HexNAc	*N*-acetylglucosamine
TMB	Tetramethylbenzidine
PTM	Post-translational modification
pH	Power of Hydrogen
EtOH	Ethanol
rt	Room temperature
g,G	Gram
ng	Nanogram
M	Molar
L	Liter
mL	Milliliter
µL	Microliter
nm	nanometer
kDa	Kilodalton
OD	Optical Density

SDS-PAGE	Sodium Dodecyl-Sulfate Polyacrylamide Gel Electrophoresis
Mw	Molecular weight
e.g.	Exempli Gratia
et al.	Friends
Rpm	Revolutions per minute
min	Minute
v	Volume
w	Weight
U	Unit
%	Percent
^{o}C	Degrees Celsius
h	Hour
V	Volts
s	Second
MS	Mass spectrometry
2-AA	2-Aminobenzoic acid
MALDI-MS	Matrix Assisted Laser Desorption/Ionization- Mass spectrometry
DMSO	Dimethyl sulfoxide
ACN	Acetonitrile
TFA	Trifluoroacetic acid
DHB	2,5-Dihydroxybenzoic acid
$NaCNBH_3$	Sodium cyanoborohydride

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CHAPTER 1
INTRODUCTION

Glycosylation belongs to the prevalent kinds of post-translational modification that polypeptides may undergo (Zhu et al., 2017). The process of formation of glycans attached to protein by binding of saccharides with glycosyltransferase enzymes composes substantial post-translational modification (Ohtsubo and Marth, 2006). As a result of glycosylation, added glycans to proteins play significant roles in modulation of immune responses, protein recognition, folding and targeting. Glycans; polysaccharides are carbohydrate polymers formed by alive beings (Yang et al., 2019). They are structurally diverse and the most abundant and biopolymers, which can be exist in the form of free oligosaccharides or glycoconjugates. The diversity of glycan structure differs according to the folding of peptide and their recognition by glycosyltransferase enzymes. Glycans contribute to cell-cell communication. The principally studied glycosylation type at glycoproteins, taking place in Golgi apparatus and endoplasmic reticulum is *N*-glycosylation (Aebi, 2013).

Milk comprises a crucial part of a balanced diet because of its various worthy constituents. The health benefits of milk concerned with the abundance of proteins, however milk is a vital source of nutrition not just because of its nutritional worth but also because of its biological characteristics.. For example the anticarcinogenic activities, the modulation of immune system and other characteristics of milk depends on its proteins and bioactive peptides (Davoodi et al., 2016). Milk proteins undergo post-translational changes that not only increase complexity of their composition but also support their biologic roles. For better understanding the biological value of its bioactives, as well as to choose the nutritionally wealth milk which accounts for the basic nutrient especially for newborns and also for youngs is important to determine the bioactive milk *N*-glycans in different cattle breeds.

Within the scope of this thesis study;

- The total milk proteins were isolated and their protein profiles were monitored in order to indicate the changes between different cattle breeds.
- The Lactoferrin (LF) and the Immunoglobulin G (IgG) amount which constitutes rich *N*-glycan sources for human health were determined.

- Glycans attached to glycoproteins were released using Peptidyl-*N*-glycosidase F (PNGase F) enzyme and purification.
- Determination of the total carbohydrate content by Phenol Sulfuric Acid Assay.
- Quantification of purified glycans by MALDI-MS.

1.1. Post-translational Modifications (PTMs)

Synthesis of a protein happens through a process called translation. Post-translational modification (PTM) consisting of thereafter phases of protein synthesis, which are changes made to a polypeptide chain chemically following the transcription of DNA to RNA and translation to protein (Uversky, 2013). These chemical changes extend from enzymatic cleavage of a peptide bond to covalent addition of small molecules or polypeptides to the amino acid side (Pang and Wilkins, 2018).

Post-translational modifications are crucial for protein folding, stability, managing the nascent protein to certain cellular spaces, targeting and their interaction with ligands (Uversky, 2013). It is known that; almost 80% of mammalian cells undergo post-translational modification. According to studies more than 400 different types of post-translational modifications (Khoury et al., 2011) influence many aspects on protein functions. The widespread kinds of post-translational modifications comprise methylation, ubiquitination, phosphorylation, acetylation, glycosylation (Ramazi and Zahiri, 2021) (Figure 1). Glycosylation being one of the major factor at biochemical processes, have important role in protein folding, stability and localization.

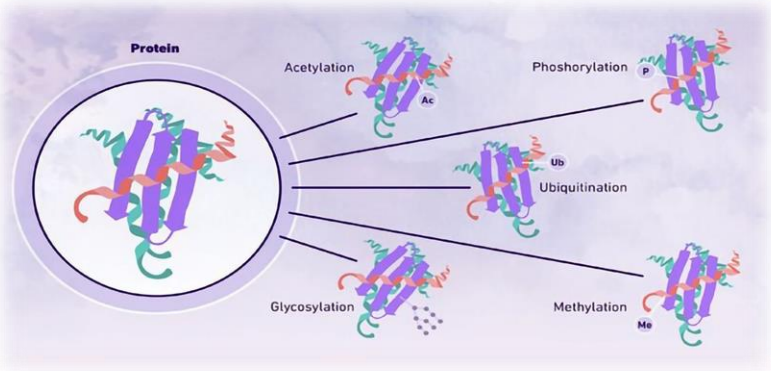

Figure 1. Post-translational modification types (Campbell,2020).

1.2. Glycosylation and Its Importance

Glycosylation existing as a complex post-translational modification in cell, is a type of reversible enzyme-directed reaction (Karve and Cheema, 2011). Glycosylation happens in different subcellular compartments, such as Golgi apparatus, endoplasmic reticulum (ER) (Stanley, 2011) and cytosol. It occurs in secreted proteins, and at prokaryotic and eukaryotic membranes.

Classification of glycosylation types depends on the target residues. These can be sorted to 6 categories. Phosphoglycosylation, glypiation (GPI-anchored), *C*-glycosylation, *N*-glycosylation, *O*-glycosylation, and *S*-glycosylation, (Blom et al., 2004). The most commonly studied glycosylation forms are *O*-glycosylation and *N*-glycosylation. Proteins are attached to glycans by glycosidic bonds and form N- or O-linked glycans (Karav et al., 2015; Parc et al., 2017).

Pursuant to researches, in various biological proceedings including molecular trafficking, cell adhesion, receptor stimulation, interaction between cell to matrix and cell to cell, protein degradation, intracellular trafficking and secretion, protein folding and signal transduction, the role of glycosylation is significant (Caragea et al., 2007).

1.3. *N*-glycans

N-linked glycans (*N*-glycans) are bound to proteins' asparagine residues with *N*-acetylglucosamine bonds (GlcNAc), AsN-X-Ser/Thr (except the proline any other amino acid can be acceptable for X) in specific amino acid sequences (Varki, 2015). Core of *N*-linked glycan is made up from three mannose residues and two GlcNAc, while the *N*-glycan core at the HexNAc residue can contain fucose (Varki, 2017). Depending on the abundance of fucosylated and sialylated *N*-glycan domains sorted in 3 groups; hybrid, high mannose, and complex-structured (Varki and Gagneux, 2017). Furthermore, for the recognition of microorganisms by cell membrane lectins and their association is mediated by *N*-glycans. They play the major role (Nwosu et al., 2012). In addition to this, glycans are essential for proteins' structural and behavioural features (Spik et al., 1994).

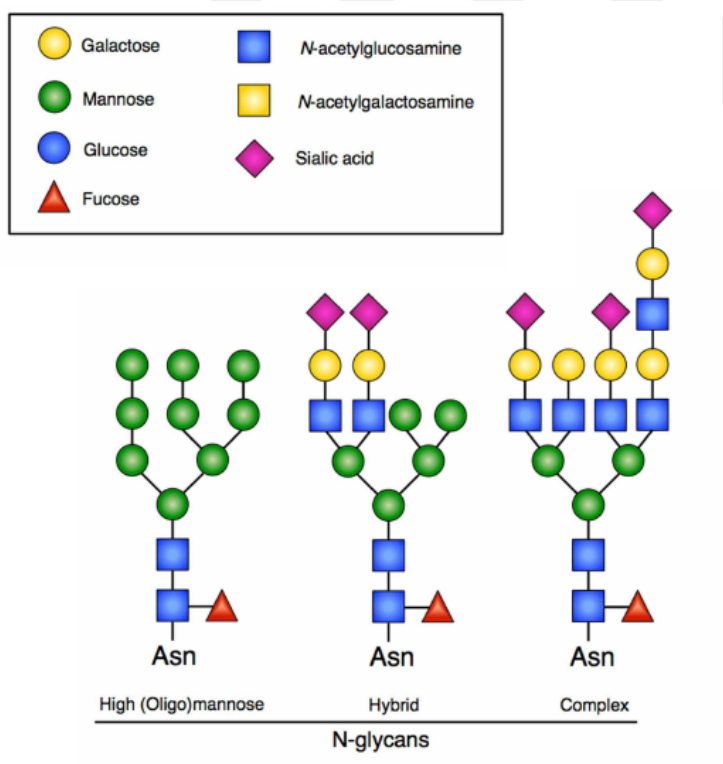

Figure 2. *N*-glycan types (Lyons et al., 2015)

1.4. Deglycosylation Strategies

For the investigation of glycan function and composition, they require release from glycoproteins. There are various deglycosylation strategies to figure out the glycan contribution to glycoprotein function. These are chemical and enzymatic methods.

Chemical methods due to their low cost, easy applicability are extensively applied methods (Sojar and Bahl, 1987). Hydrazination and beta elimination are the chemical methods for release of a glycan from glycoproteins. Besides of being widely used methods, they have some limitations. For example, the used chemicals during processes have carcinogenic effects and the remained sodium at the end of the reaction obstruct the glycan analysis in a mass spectrometer (Choudhary and Hansen, 1998). Nevertheless, applying hydrazination method emerges some limitations. This method is applied by altering the reaction parameter in order to release glycans and this parameter is the temperature. Hence, these chemical methods due to their limitations are not suitable to be used in food and health areas, as well as they change the glycoprotein structure.

On the other hand, to eschew these problems there are different enzymatic strategies applied for the release of glycans. For example, Peptidyl *N*-glycosidases and specifically Peptidyl *N*-glycosidase F in short PNGase F as a member of asparagine amidase class (EC 3.5.1.52) (Loo et al., 2002) is the most widely used enzyme in glycoanalytical processes because of their ability to separate from glycoproteins various *N*-glycan structures with greatest specificity (Vilaj et al., 2021). PNGase F enzyme hydrolyses at the glycosylamine linkage and also enhances the generation of an intact oligosaccharide and a peptide devoid of carbohydrates when combined with di-*N*-acetylchitobiose (Tarentino et al., 1985). From glycoproteins the separation of *N*-linked oligosaccharides is suitably carried out by PNGase F enzyme. It can cleave *N*-glycan chains in GlcNAc and asparagine residues of comlex, hybrid, and high mannose complex carbohydrates. PNGase F cannot recognize the carbohydrate if the core have an α-1,3-linked fucose remaining part, (Figure 3) mostly they present in glycoproteins of insect and plants (TRETTER et al., 1991).

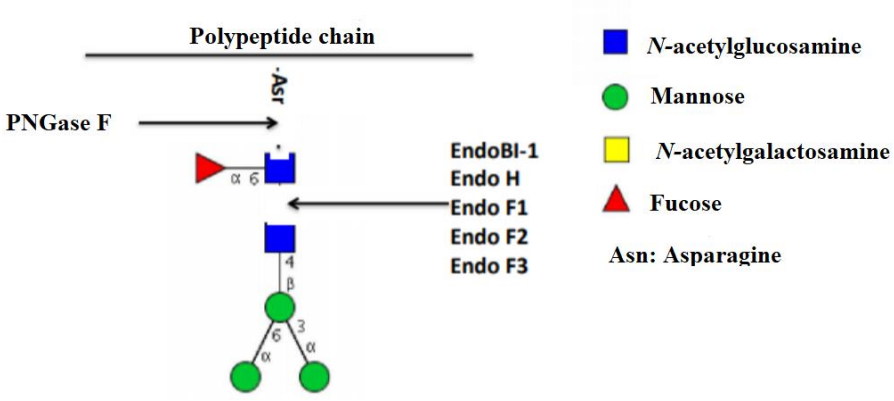

Figure 3. Cleavage sites on *N*-glycan of PNGase F and other endoglycosidases

1.5. History of Milk

Consumption of dairy products seems to have been introduced by Europeans populations about 11,000 years ago. Until then, milk was strong toxic product to humans as they were unable to produce the enzyme which is responsible for the breakdown of lactose (lactase). However, as the crop of land and the breeding of domestic animals began to replace hunting in the Central Europe, breeders have devised ways to reduce milk content to this sugar through its fermentation. After a long time, the genetic mutation that expand in Europe, resulted in production of the enzyme lactase and thus enabled humans to consume large amounts milk. Since then milk has been a very important part of their diet of people because of its caloric value but also the many beneficial ingredients that contains made it a major (Curry, 2013).

1.5.1. Description

Along a stage of rapid growth and development, epithelial cells in mammary gland which are special produces milk to provide nutrition for one immunologically immature new-born. (Walzem et al., 2002). Ruminant milk is of course excreted for nutrition of their own new-borns, but nevertheless was, is and will be crucial component in human nutrition, as it contains a wide range of nutrients, and most of them are in significant concentrations. It provides components crucial for their nutrition, immunity and development.

Milk is a blend of many ingredients, most notably water, which can varies in concentration. Especially cow's milk consists of an average of 87% water, 3-4% fat, 3% protein, 4-5% lactose, 0.1% vitamins and 0.8% minerals (Pereira, 2014).

1.6. Factors Affecting Milk Composition

Significant elements affecting the composition of milk:

- Lactation period
- Lactation count
- Breed
- Influences of season and environment

Amount of protein in milk depends on the genetic factors about 55% and the 45% are other factors which can be changed by factors like lactation stage and the health of udder. The percentages of protein and milk fat varies between hot and cold weathers. For examle in cold weather is high but in hot weathers during spring and summer this percentage is low. This alteration in percentages is affiliated to the climatic conditions and availability of feed (Varga and Ishler, 2007). Feeding, a balanced diet applied to cows have remarkable impact both on milk protein and fat amount. Breed has substantial impact on milk yield but its effect on content is less. (Nickerson, 1995).

1.7. Milk Glycans

Glycans rich in milk may found in glycoconjugate or in free forms. Free glycans in milk also known as oligosaccharides, formed by *N*-acetylglucosamine (GlcNAc), fucoses and sialic acids (Varki et al., 2009). Milk glycans are related with valuable effects protecting newborns from infections and the regulating their immunity. They have impact on how an infant develops and how they interact with their gut microbiota.

Glycans included in milk have various essential roles with a large extend of interest. The interactions of glycans happen unmediated with host-tissue or may happen via host-

associated microbes. The coaction happened via the host-associated microbes can lead to indirect impacts, where the host functions are influenced by the glycan-mediated microbial activity. For example some of the human milk oligosaccharides (HMOs) structure-function relationships can be presented like; protection against pathogens and enhancement of intestinal microbiota (Smilowitz et al., 2014). In addition to these, HMOs influence immune system and modulate the intestinal epithelial cell responses (Bode and Jantscher-Krenn, 2012). The biological role of bovine milk oligosaccharides (BMOs) seems to be similar with the biological functions of the HMOs as was referred above. BMOs and bovine milk glycoconjugates hinder the pathogens to adherence on gut cells. Furthermore, BMOs have significal roles in health enhancing areas, such as reducing inflammation by decreasing gut permeability. Milk glycans confer protection against pathogens and prevent the adherence of bacterial toxins to receptors in host cell (Morrow et al., 2005).

In many studies BMOs have been identified as potentially functional components which can be handled in industrial areas like, can be used in infant formulas, at nutraceutical and therapeutic products (Zivkovic and Barile, 2011). Additionally the bovine milk oligosccharides may have a mitigating role in some diseases, like including metabolic and inflammatory disorders in infants and adults (Sunds et al., 2021). The consumption of BMOs in animal studies have shown that these oligosaccharides may reduce the leaky gut syndrome condition (Boudry et al., 2017). According to the studies, oligosaccharides found in milk of bovines are predominantly sialylated, that the 70 percent of the structures observed in first milk and 50 percent of composites belongs to matured milk includes sialic acid (Tao et al., 2009).

1.8. Milk Proteins and Their Importance

Milk contains proteins of high biological value, for example, proteins which the human body can utilize to a great extent degree for the synthesis of its proteins. Due to of their easy digestibility and their biological value, milk proteins constitute an important source in terms of nutritional physiology. Milk proteins contribute to the development and growth of organisms because they serve as building blocks. At the same time, they are the

major energy sources. Their nutritional value is coming from the entity of essential amino acids (Kalyankar et al., 2015).

Milk proteins can be categorized into two groups:

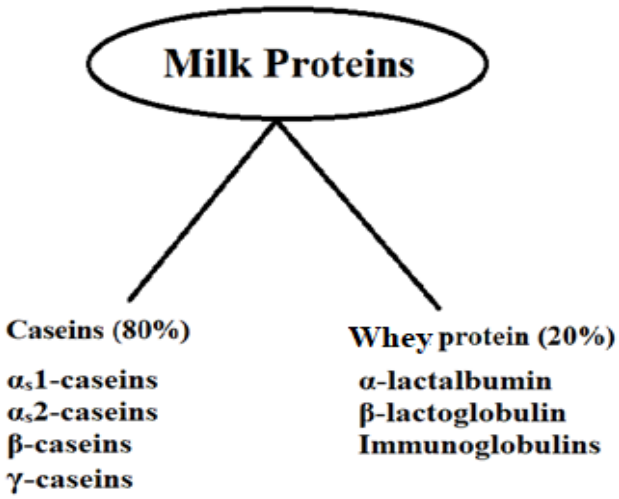

Figure 4. Classification of milk proteins (Kalyankar et al., 2015).

Bovine milk proteins including the fundamental amino acids are states as high-quality or complete proteins. Besides of having nutritional benefits, milk proteins also used as therapeutic agents. In latest years, researchers have been searched for milk proteins' properties, were investigated whey proteins' function in inhibition of incidence and growth of chemically induced tumours (Davoodi et al., 2016). In addition to this, milk proteins can be used as coadjuvant in traditional medicine to treat cardiovascular disease conditions, intestinal health and metabolic problems (Hsieh et al., 2015).

1.8.1. Whey Proteins

Whey proteins are resolvable milk proteins known as serum proteins found as globular proteins. They form the 20% of the whole milk proteins. Containing ,α-lactalbumin, and β-lactoglobulin (Kilara and Vaghela, 2018). Due to of the content of amino acid, whey proteins recognized to offer high nutritional value. Over and above this, whey proteins possess various functional and biological properties affecting different biological functions supporting the growth of bones and strengthing muscles, decreasing cholesterol and enhancing the mood and cognitive activities (Krissansen, 2007). Furthermore, they can be used in anti-oxidative, anticancer, immunomodulatory and anti-inflammatory functions. Because of their health promoting activities, whey proteins are searched for utility alone or with other potential drugs against involving virus infection (Hazra et al., 2021).

1.8.2. α-Lactalbumin

In human milk is the mainly found protein, constitutes the 20% of bovine whey proteins'. It is metalloprotein having molecular weight ~14 kDa. Pretty much at all mammalian species' milk the production of lactose is regulated by α-Lactalbumin (Qasba and Kumar, 1997). Biological role of α-La is to contribute to the lactose synthetase process (Goulding et al., 2019).

1.8.3. β-Lactoglobulin

β-Lactoglobulin is globular protein found in many mammalians' milk, except human milk (Goulding et al., 2019). Bovine Lactoglobulin has been used as model protein for many biophysical tests of self-association, stability, folding because of its availability and relative simplicity in purifying (Crowther et al., 2016). β-Lactoglobulin according to studies is a cow's milk allergen (Fei et al., 2020). Therefore, the newborns' gastrointestinal tract is immature and a small amount consumption of milk containing β-Lactoglobulin can trigger the mucosal and systemic immunity (Vocca et al., 2011).

1.8.4. Immunoglobulins

Antimicrobial protection against microbial infections maintained by the immunoglobulins of bovine colostrum which offer passive immunity to young calf until its immune regulation completed (Korhonen et al., 2000a). Immunoglobulins (Ig) are glycoproteins produced by plasma cells. They are member of globular proteins possessing antimicrobial and protective properties. IgG antibodies have various functions, where the most essential function is the stimulation of complement-mediated bacteriolytic actions. Another important role is to improve the identification and phagocytosis of bacteria by leucocytes. Furthermore, can impede bacterial metabolism, deactivate viruses and toxins as well as prevent microbes from adhering to surfaces (Korhonen et al., 2000b). Because of their biological properties, also they can be used as both therapeutically and prophylactically (Sedlacek et al., 1983).

They can be found in various quantities in milk, colostrum and in blood serum (Gapper et al., 2007). The immunoglubulins can be sorted into 5 classes; IgA, IgG, IgM, IgE, and IgD; The major immune system supporting protein IgG provides parennial immunization. The IgG is made by two heavy and two light chains (Janeway et al., 2001). For the remove of pathogens is not initiates the complement cascade only, but through neutralization it protects from viral infections at the level of mucosa (Baba et al., 2000).

1.8.5. Lactose

Lactose being the major component in human and animal milk, behaves like an energy carrier, especially during infancy years (Vesa et al., 2000). It is a disaccaharide consisted of galactose and glucose. Lactose mainly found in milk of mammals, so lacks in plant-based. The lactose amount of bovine milk is approximately 4.8%. Furthermore, the lactose content does not differ a lot among dairy cattles, it changes as the lactation stage increases and occurs mastitis contamination (Fox, 2011). According to studies, important functions of lactose is the absorbtion of calcium and other minerals during infancy (Ziegler and Fomon, 1983). Also, it can be used by the intestinal microbiota as nutrient (Parche et al.,

2006), which it is not digested in small intestine. The growth of bifidobacteria promoted by lactose which play a life-long role in countering the ageing linked decrease of some immune functions(Venema, 2012).

1.8.6. Lactoferrin

Lactoferrin (LF); single-chain glycoprotein having 76 kDa molecular weight consisted of 692 amino acids (Moore et al., 1997). It has the ability to bind and transfer iron ions. It has been firstly discovered by Sorensen and Sorensen in 1939 from bovine milk and subsequently in 1960 was specified by Johanson (Johanson et al., 1960).

Lactoferrin is a molecule which has been discovered in different mammalian species milk like cow, buffalo, goat. Foremost lactoferrin is available in high concentrations in breast milk and serve as an iron-binding glycoprotein (Steijns, 2001). Besides of serving as an iron-binding glycoprotein it offers a range of biochemical and bilogical properties such as antioxidant, antibacterial and presents antiviral activities. In addition to these, lactoferrin is substantial for immune host defense mechanism (Karav et al., 2017). At latest studies was stated the vital of lactoferrin assisting the development of immune system abilities in newborns and infants (Manzoni, 2016).

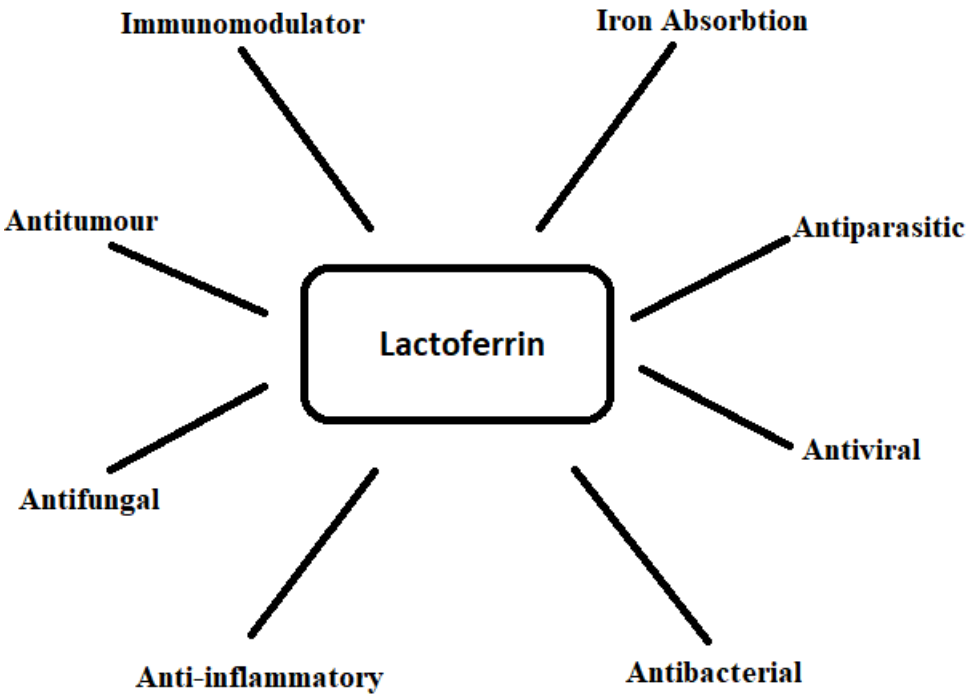

Figure 5. Properties of Lactoferrin (Brock, 2002).

Due their benefits and its antioxidant and antimicrobial properties, lactoferrin has many applications in industry as a food ingredient. Bovine lactoferrin is used to enhance foods like cakes, pastries, yogurts, and drinks, as well as cosmetics (Karav et al., 2017). Also the European Food Safety Authority (EFSA) directed evaluation of bovine lactoferrin as nutritional ingredient in diverse products, like in infant formula (Jhaveri and Arya, 2015).

In current studies, was studied the use of lactoferrin in several forms and sources, which will be used to treat various conditions. For example the bioavailability of lactoferrin usage as a nutraceutical considered to be major for impeding or cure of COVID-19 (Kell et al., 2020).

As a result supply of lactoferrin from mouth has several useful health profits in humans, animals, involving anti-inflammatory, anti-cancer actions (Teraguchi et al., 2004).

1.8.7. Lysozyme

Lysozyme (Lys) being one of the minor milk proteins, attract the attention of majority because of its efficacious antimicrobial activity against various microorganisms and its use in the area of food preservation and safety (Benkerroum, 2008). Lysozyme performs its antimicrobial activity by hydrolysing the glycosidic bonds of mucopolysaccharides in cell walls of bacteria. Lysozyme jointly to else peptides for the decline of gastrointestinal infections found in active form in the digestive tract of infants (Polidori and Vincenzetti, 2012).

1.8.8. Lactoperoxidases

The Lactoperoxidase (LPO) enzyme found in mammalian milk, saliva and tears is a heme protein and glycoprotein (Ozdemir et al., 2001). Bovine milk contains Lactoperoxidases abundantly, about 30 mg/mL, making up the 0.5% of the whey protein. Lactoperoxidases are recognized as innate antimicrobial systems in human secretions (Kussendrager and Van Hooijdonk, 2000). In many studies have been indicated that the LPO can modulate the oral microbiota of breastfed newborns. Furthermore, LPO is commonly utilized for dairy product conservation, especially in the absence of refrigeration, due to its antibacterial action (Ndambi et al., 2008).

CHAPTER 2
PREVIOUS STUDIES

Bovine milk including the food substances stand for growth and development of infant, serves as weld of lipids, vitamins and proteins. Also it comprises hormones, growth factors, immunoglobulins and bioactive peptides being a major nutrient for newborn. The composition of milk changes according to lactation season of cattle, breed, the health condition of udder and the nutrient supplied for cattle are the significant elements impacting the composition, because it has dynamic nature. Throughout the lactation season, the content of milk appears to alter in response to the varying demands of developing newborn, providing different quantities of ingredients significant for growth and development, non-specific and specific host defence (Haug et al., 2007).

As the milk serves as a major nutrient for the development of neonates due to its rich composition, made one of the mostly searched subjects. Especially there is an interest about its components. In many studies have been investigated the compositional differentiation at milk and colostrum of bovines. Beside of compositional differences observed in bovine colostrum and milk (Abd El -Fattah et al., 2012), also has been remarked changes at glycosylation sites. Beyond the material used such as colostrum or milk, also the breed factor can influence the composition. The milk yield at Holstein-Friesian breed is high, nevertheless the protein amount was poor. On the other hand at Jersey and Brown Swiss breeds the milk production is poor but the content of protein and fat are rich (De Marchi et al., 2008).

In 2010 was analyzed the Immunoglobulin G and Lactoferrin levels in milk of different bovine strains, where chosen the Simental, Polish Holstein-Friesian Black-White and Red-White variety, Jersey. In this study was indicated that the breed factor affects the concentration of bioactive proteins. The milk belonging to Simental and Jersey cows contain great amount of lactoferrin (116.74 and 103.48 mg/L), and immunoglobulin G (579.9 and 508.6 mg/L) compared to other breeds (Król et al., 2010).

Also in another study was stated the amount of antimicrobial proteins associated to immune system of Holstein and Jersey cows which differs between each other. It was revealed the lactoferrin amount of Holstein breed was greater from the breed Jersey (Tacoma et al., 2016).

In addition to animal genetics and diet also other factor such environmental, and lactation stage can alter the physiology of animal and its milk composition. It was stated that the seasonal changes can influence the composition of milk in Holstein breed, where there is decrease at protein (3.5 to 3.3%), fat (3.8 to 3.2%) concentration, from cold to hot weather (Bernabucci et al., 2015).

In 2019 the research which was performed to bring to light the significant changes in oligosaccharide plenitude between Jersey dairy cattles and Danish Holstein-Friesian was investigated the oligosaccharide content of milk from different cattle breeds and were found that milk of Jersey comprises higher quantities of fucosylated, sialylated oligosaccharides (Sundekilde et al., 2012). In this study was examined 20 samples from 2 different breeds and was concluded that most milk oligosaccharides were found in significant amounts plentifully in Jersey milk in consideration to Holstein-Friesian. Nevertheless, in Jersey breed's milk was observed high cow-cow variability in the abundance of fucosylated oligosaccharides. Besides their differences in the abundance of oligosaccharides, also the milk obtained from two different cattle breeds shared similarities in the structure and composition. In both breeds were observed 15 oligosaccharides (Robinson et al., 2019).

In this study, will be examined the bioactive milk components from four different cattle breeds situated in Canakkale, Turkey, in order to better understand how some factors like breed, will influence the protein amount, the concentration of IgG and lactoferrin and the glycan abundance in each breed.

CHAPTER 3
MATERIAL and METHOD

3.1. Material

Under this subsection will be mentioned all the necessary items to complete this thesis experiments, including chemicals, substrates, and the used laboratory equipment.

3.1.1 Chemicals, Kits, and Required Items

In Table 1, the chemicals, kits, and essential items required for the given study were listed.

Table 1

List of chemicals, kits, and required items

Name of Materials	Catalog Number	Items
Phenol	P1037	Phenol sulfuric acid assay
Sulfuric acid (95-98%)	112080	Phenol sulfuric acid assay
Acetic acid	137130	SDS-PAGE analysis
Methanol	106012	SDS-PAGE analysis
Ethanol	920.026.2500	Protein isolation
Bovine IgG ELISA Kit	LS-F10111	Determination of IgG concentration
Bovine LF/LTF/Lactoferrin ELISA Kit	LS-F4884	Determination of lactoferrin concentration
Coomassie Brillant Blue R 250	0472-25G	SDS-PAGE analysis
SureCast™ Acrylamide Solution (40%)	HC2040	SDS-PAGE analysis
SureCast™ TEMED	HC2006	SDS-PAGE analysis
Ammonium Persulfate	17874	SDS-PAGE analysis
Isopropanol	961.023.2500	SDS-PAGE analysis
Sodium dodecyl sulfate (SDS)	SDS001.500	SDS-PAGE analysis
10X Running Buffer (Tris-Glycine SDS)	TGS10	SDS-PAGE analysis
Laemmli Sample Buffer (2X)	LSB-2x	SDS-PAGE analysis
Tris base	TRS001.1	SDS-PAGE analysis
Qubit™ Protein Assay Kit	Q33211	Determination of protein concentration
Glycobuffer 2,	P0704S	Buffers for glycan release

Glycoprotein Denaturing Buffer, NP-40		
L-Rhamnose monohydrate	83650	Induction of protein expression
HisPur^{TM} Ni-NTA Resin	88222	Protein purification

Table 1 (continue)

PNGase F (N-His SUMO)	FHD-2021-3491	Release of glycans from glycoproteins
Tris	TRS001.1	Cell lysis
Imidazole	56750	Cell lysis
Sodium Chloride	31434-5Kg-R	Cell lysis
Sodium Phosphate Monobasic Anhydrous	0571-1Kg	Cell lysis buffer
Sodium Phosphate Dibasic Dihydrate	04272-1Kg	Buffer for cell lysis
10-kDa-cut-off centrifugal filter	UFC9010	Enzyme concentration

3.1.2. Substrates

Milk samples used for the analysis were indicated below:

- Holstein
- Jersey
- Brown-Swiss
- Guernsey

They were provided from a dairy farm of Çanakkale, Uluova Milk Trading Co.

3.1.3. Laboratory Equipment Used for Molecular Analysis

All laboratory equipment required for the thesis studies was handled and maintained according to the user's instructions in the research lab of Molecular Biology and Genetics Deparment at Çanakkale Onsekiz Mart University (ÇOMU). The equipments required for study were listed in Table 2.

Table 2

List of Laboratory Equipment

Device name	**Brand name**
Cooling centrifuge	Hettich Mikro 200 R
Centrifuge	Beckman Allegra X-15R
Speedvac evaporator	Labconco
Orbital shaker	STUART
Analytical balance	Shimadzu
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	INOVIA
Pure water system	Millipore
Power supply	Bio Rad
Bruker rapifleX™ MALDI Tissuetyper™	Bruker Daltonik GmbH, Bremen, Germany

3.2. Methods

Within the scope of this subheading will be referred the experimental steps used for this study and the methods which were used are mentioned in detail.

3.2.1. Protein Isolation from milk samples

Milk samples for this study was acquired from regional dairy farm of Çanakkale, Uluova Milk Trading Co. Milk sample from each cattle breed stored in 500 mL bottle (Figure 7).

Protein isolation from raw milk achieved by using ethanol precipitation. Firstly 1 mL of milk from each breed put in falcon tube and centrifuged at 4000 rpm 4^{o}C, 20 min. After centrifugation obtained 3 phases. The upper phase containing the lipid part was removed carefully using spatula and the middle phase transferred into a new falcon tube. Onto these was added 1:4 (v/v) cold ethanol and were incubated 1h at -20^{o}C. After incubation falcon tubes were centrifuged at 4000 rpm, 4^{o}C, 10 min. And supernatants were removed. The obtained pellet homogenized with 1 mL dH_2O. Onto falcon tubes added 4 mL cold ethanol and were incubated at -20^{o}C overnight. After an overnight incubation were applied centrifugation for 15 min. at 4000 rpm and 4^{o}C and supernatant removed. To suspend the remained ethanol the lid of falcon tubes were let semi-opened. The pellets were dissolved by 1 mL dH_2O and were stored in -20^{o}C for further use (Figure 8).

3.2.2. Visualization of Isolated Milk Proteins by SDS-PAGE Electrophoresis

An Invitrogen Mini Gel Tank were used for SDS-PAGE gel electrophoresis to visualize the isolated milk proteins. For the analysis of protein bands were prepared 4% stacking and 12% seperating gel (Table 3). To compare the molecular weight of proteins were used a PageRuler Plus Prestained Protein Ladder. The samples were put into 500 µL eppendorf tubes and onto these were added 2x Laemmli Sample Buffer in a 1:1 ratio. Then for the denaturation of proteins incubated 5 min at 95°C. For the homogenization of the mixture were used a vortex. After the incubation the gel tank completed with 1X Tris-

Glycine-SDS Running Buffer for running of proteins in the gel. Protein ladder and the samples were loaded and were run for 1 hour at 200 V. The seperated proteins according to their molecular weight were stained at 55 rpm for 30 min with Coomassie Brilliant Blue Dye and they were destained with destaining solution by changing at 30 min until the band observation ensured.

3.2.3. Determination of Protein Concentration

The amount of protein isolated from milk samples quantified by QUBIT 3.0 Fluorometer (Thermo Fisher Inc, CA USA). After instrument was calibrated with appropriate standards, was added 197 µL Qubit™ protein buffer, 1 µL Qubit™ protein reagent and 2 µL sample in tube and was incubated in dark at room temperature (rt),15 min. As incubation completed was applied fluorometric measurement and the concentration of protein was determined as well as they were multiplied with dilution factors. All the measurements were performed in duplicate.

3.2.4. Release of *N*-glycans From Glycoproteins by PNGase F Enzyme

Release of *N*-linked glycans from milk proteins achieved by Peptidyl *N*-glycosidase F (PNGase F) enzyme which was recombinantly produced in BAP project (FHD-2021-3491).

Enzyme Production and Purification

E. cloni 10 G cells containing the gene region of the enzyme desired to be produced were inoculated into 33 mg/mL Lysogeny Broth (LB) medium containing Kanamycin and incubated at 37°C, 160 rpm till the cell's OD reach approximately to 0.5-0.6 OD_{600nm}. Cells stimulated for protein production by adding 20% stock rhamnose solution with 0.1% of final concentration to the cells whose cell density reached the expected level. After addition of Rhamnose solution the cell culture were incubated at 24°C, 160 rpm overnight. After an overnight incubation the induced cultures centrifuged 15 min at 4000 rpm, 4°C . The obtained pellets incubated at -80°C for 15 minutes and they were prepared for cell lysis.

Firstly the pellets were homogenized by adding 5mL dH_2O and then they were centrifuged 4000 rpm, 4°C for 15 min. After centrifugation supernatant was throwed away and on remained part 6300 μL lysis buffer were added and incubated in ice for thirty minutes. As a second lysis process was applied sonication for 10 seconds. After sonication centrifugation process was applied in order to move away the cell debris.

Purification method was achieved by using Batch method. Initially two resin-bed volumes of equilibration buffer in the Ni-NTA resin was added and was equilibrated. Supernatant fraction obtained after centrifugation were collected in a single sterile falcon and mixed 1:1 with equilibration buffer and on resin containing falcon tube was added. The obtained mixture was shaken for 30 minutes under the conditions of 24°C, 200 rpm in order the proteins bind more tightly on resin. After incubation the sample was centrifuged at 700xg, at 4°C, 2 min and supernatant was removed. Onto remained resin was added approximately 15-20 mL wash buffer and was applied centrifugation at 700xg, 4°C for 2 minutes to remove the potential impurities. This process was repeated 3 times. After the wash step was added 10 mL elution buffer to elute the proteins bound to resin and was centrifuged at 700xg, 4°C for 2 minutes. Later on, to concentrate the supernatant used Amicon tube (10-kDa cut-off). The concentrated enzyme was stored at -80^{o}C at small aliquots until use.

Enzymatic Reaction for the Deglycosylation of Glycoproteins

The enzymatic reaction conditions were applied by combining the glycoprotein with Glycoprotein Denaturing Buffer (10X) and denaturing them at 100^{o}C for 10 minutes by heating reaction. After that adding 10% NP-40, Glycobuffer 2 (10X), PNGase F enzyme on reaction tubes and distilled water to control tubes, they were incubated at 37^{o}C for 3 hours. After incubation for the conformation of glycan seperation from milk glycproteins were applied SDS-PAGE gel electrophoresis. The samples in a 1:1 ratio were mixed with Laemmli and were incubated 5 min at 95°C. Then, the samples were loaded to the gel and they were run at 200 V for 1 hour. After stained by using Coomassie Brilliant Blue Dye and with destaining solution the dye was washed, and natural and deglycosylated proteins on gel was observed.

3.2.5. Glycan Quantification

After the release of glycans, the deglycosylated proteins were precipitated by cold ethanol in a 1:4 ratio (v/v) and were incubated at -20ºC for 1 h. After incubation for 3 min at 12.000 g applied centrifugation. Upper part which contains glycans were taken and were transferred into a new eppendorf tube. They were dried at vacuum evaporator for 1 hour. After the drying process was completed, each purified glycan were dissolved with appropriate amount of distilled water and were kept at -20ºC for further usage.

Determination of Total Carbohydrates by Phenol Sulfuric Acid Method

The release of glycans from milk glycoproteins was evaluated by performing phenol-sulfuric acid assay. Firstly were prepared the standards. First of all, 1 mg/mL glucose solution was prepared and serial dilution was performed in order to obtain 1000, 800, 600, 400, 200 and 100 µg/mL of glucose standards as final volume is 0.1 mL. 25 µL of each standard as dublicates were put into 96 well plate and 25 µL samples were put as dublicates. Then, from 5% phenol solution was added into wells 25 µL and were mixed by doing up-down by pipette and finally 125 µL concentrated sulfuric acid was added and by pipetting were mixed immediately. Then, 96 well plate were incubated at 35 rpm and least 30 min in dark. After incubation measurement of OD was carried out at 490 nm and the standard curve was plotted.

3.2.6. Determination of Lactoferrin Concentration

For the determination of lactoferrin quantity in milk used ELISA assay. The milk samples were dissoleved (50-55ºC) and 5 mL from each sample was added to sterile falcon tube and were centrifuged at 1000xg for 20 min in order to eliminate the particulates. For the assay the supernatant part was transferred into a new sterile falcon tubes and were diluted appropriately (1:1, 1:10, 1:100, 1:1000, 1:10000 and 1:100000) by sample diluent. First of all the reagents to be used for the assay were brought to room temperature. From standards and samples were added 100 µL in wells as dublicates. They were coated with sealer and at 37 °C were let for a hour incubation. After incubation, liquid part were aspirated from each

well, and from Detection Reagent A working solution were added 100 µL to wells. The plate was covered with a plate sealer and smoothly was shaked to mix well and were incubated at 37 °C for 1 h. Afterwards the liquid from each well were aspirated and performed washing process for 3 times. Approximately were added 350 µL of 1x Wash Buffer to each well by using a squirt bottle. Before a complete aspiration each wash were allowed to sit for 1-2 min. After the last wash step, to detract from the remained Wash Buffer was aspirated well and the plate were inverted on the clean absorbent paper. Subsequently, into each well were added 100 µL of the Detection Reagent B working solution and plate were wrapped with sealer and 30 min at 37 °C applied incubation. As well as the incubation was completed from wells the liquid was aspirated and 5 times washed as performed before. Thereafter, into each well was added 90 µL of TMB Substrate solution, 10-20 min at 37 °C was incubated protecting from light. Lastly from Stop Solution 50 µLwas added in wells and immediately determined the optical density (OD value) at 450 nm. By plotting the standard curve was determined the lactoferrin concentration of each breeds' milk (Figure 6).

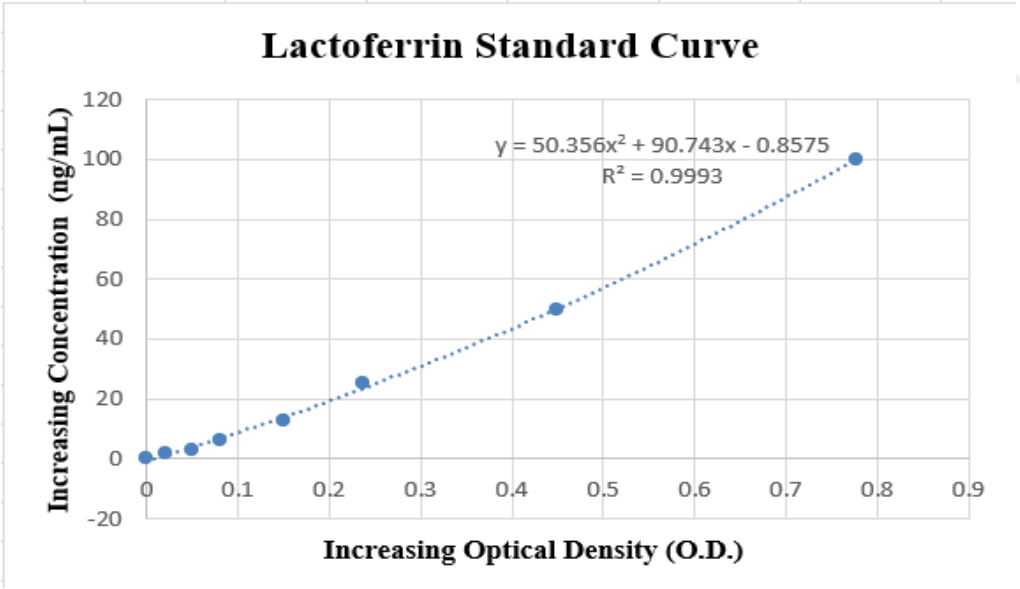

Figure 6. Lactoferrin standard curve.

3.2.7. Determination of Immunoglobulin G (IgG) Concentration

Detection of Immunoglobulin G (IgG) content in different cattle breeds' milk were performed by ELISA assay. Firstly the milk samples were centrifuged 20 min at 1000xg. The supernatant was diluted firstly with PBS to 1:500 ratio. After that applied dilution with a sample diluent. All the reagents required for the performance of assay were brought to room temperature. In each well were added 50 µL from samples and standards. Onto these were added 50 µL of 1x HRP-Conjugate solution, and the plate were wrapped with sealer. It was incubated at 37°C for 40 min. After incubation, the liquid from each well were aspirated and performed 5 times the washing process using nearly 200 µL from 1x Wash Buffer. Each wash allowed to be 2 minutes in each well before aspirating. As the washing process completed the plate were inverted onto clear paper to remove the remaining wash buffer. In consequence, 90 µl of TMB Substrate were added in wells, and the plate as well as covered with sealer, incubated at 37°C for 20 min, preserved from light. Finally was added 50 µl of Stop Solution and immediately was determined it optical density at 450 nm, 540 nm and 570 nm.

3.2.8. Labelling of Released Milk *N*-glycans with 2-AA

Denaturation of the extracted proteins dissolved in 50 µL of 1% SDS was performed by incubating the samples at 90 ^{o}C for 10 min. Afterwards, 25 µL of 2% NP-40 and 25 µL of 5X PBS were added to the solution. For enzymatic deglycosylation, 1 U of PNGase F enzyme was added and the samples were incubated at 37 ^{o}C overnight. After removing the *N*-glycans from the glycoproteins, the samples were labeled with 2-AA (2-Aminobenzoic acid) to accomplish meaningful results from MALDI-MS analysis. For this process 48 mg/mL concentration of 2-AA (in DMSO/acetic acid, 7:3, v:v) and 63 mg/mL (in DMSO/acetic acid 7:3, v:v) sodium cyanoborohydride ($NaCNBH_3$) solutions were prepared. 50 µL of 2-AA and 50 µL $NaCNBH_3$ were taken from this solutions, respectively, and added to samples in which glycan release was carried out. Labeling was achieved by incubating the solution at 65 ^{o}C for 2 hours with the aid of thermomixer.

3.2.9. *N*-glycan Purification

2-AA labeled *N*-glycans were first purified by solid phase extraction containing cellulose. 150 µL of 2-AA labeled samples were added from 100% ACN in a final volume of 85% ACN. Approximately 10 mg of cellulose was transfered to solid phase extraction cartridges. Afterwards the cartridges were first washed 2 times with 1 mL of 100% dH_2O and 85% ACN. Samples were added to the cartridges and allowed to interact with the material for 5 min. Afterwards, the cellulose-containing cartridges were washed three times with 85% ACN containing 1% trifluoroacetic acid (TFA) and 85% ACN solutions. In this way, it was ensured that excess labels and other chemicals were removed. After this process, the elution of 2-AA labeled *N*-glycans was carried out with 0.75 mL water. After adding 0.75 µL of TFA to the samples, purification was started with porous graphitized carbon. Carbon material containing about 10 mg was taken into the cartridge. First, the carbon-containing cartridges were washed 2 times with 1 mL of 80% ACN containing 0.1% TFA and water containing 0.1% TFA. Afterwards, the cellulose-purified samples were added into carbon-containing cartridges. Samples were incubated for 5 min for interaction. In sequence the carbon-containing cartridges were washed 5 times with water including 0.1% TFA. Elution of 2-AA labeled glycans was performed with 0.75 mL of 80% ACN containing 0.1% TFA. Finally the samples were dried with a fast vacuum dryer at 45 ^{o}C.

3.2.10. Analysis of *N*-glycans by MALDI-MS

MALDI-MS analysis of 2-AA Labeled *N* glycans were performed using a RapiFlex MALDI-TOF/TOF-MS/MS (Bruker Daltonik GmbH, Bremen, Germany) incorporating SmartBeam 3D laser technology. 1 µL of the purified sample solutions was dropped directly onto the MALDI target plate and allowed to dry. 1 µL of 5 mg/mL DHB [2,5-dihydroxybenzoic acid (2,5 DHB)] matrix prepared using 50% water and 50% ACN was added to the dried samples. Spectra were recorded at 20 kV acceleration potential in negative ionization mode and in the mass range of 1000-5000 Da using reflectron mode. Spectra were obtained by collecting 8000 laser pulses at 2000 Hz frequency.

3.2.11 MALDI-MS Data Analysis

After the obtained MS spectra were transferred to Protein Scape V4 (Bruker Daltonik GmbH, Bremen, Germany) software, their composition was determined using GlycoQuest software. A list of detected *N*-glycans was generated.

CHAPTER 4
RESEARCH FINDINGS

4.1. Protein Isolation

Figure 7. Milk samples belonging to four different cattle breeds.

The protein isolation from milk samples was performed by using ethanol precipitation as was described above.

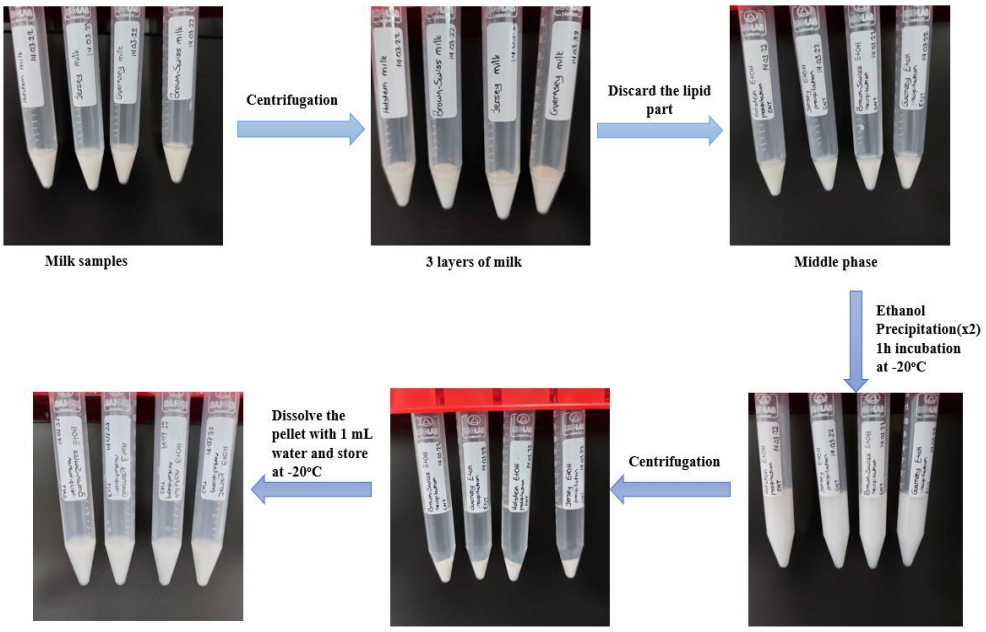

Figure 8. General scheme of protein isolation method.

4.2. Demonstration of Milk Proteins by SDS-PAGE Gel Electrophoresis

Profiles of isolated milk proteins and their molecular masses analyzed by performing SDS-PAGE gel electrophoresis. This is a prevalent assay applied for protein seperation based on molecular weights. In this study 3 µL from Protein Ladder (10-250 kDa) and 10 µL from sample loaded into the wells and they were run at 200 V 1 hour. Staining process completed, the gel was rinsed with destaining solution until the bands were observed clearly. The protein profiles of milk samples are shown in Figure 9.

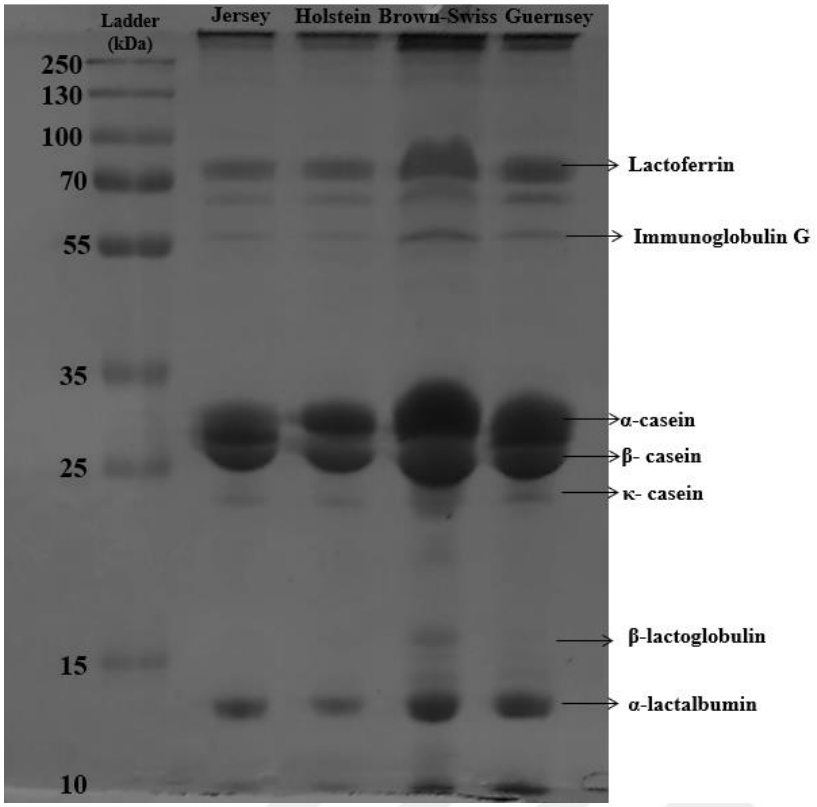

Figure 9. SDS-PAGE profiles of milk samples of different cattle breeds.

The protein profiles of milk samples based on their molecular weights were visualized by SDS-PAGE electrophoresis. Suchlike SDS-PAGE images monitoring the bovine milk proteins were obtained at previous studies (Costa et al., 2014). The bands belonging both to Lactoferrin and IgG were clearly observed at each breed. The abundance of proteins in samples can be estimated by the relative intensity of bands. High content of protein in sample indicated by thick bands and the thin bands point low amount of protein in sample. The protein bands belonging to the breed Brown-Swiss are more densely compared to bands of other breeds remarking great amount of proteins.

4.3. Detection of Protein Concentration by Qubit Assay

The protein concentration of each milk sample was detected by using a Qubit 3.0 Fluorometer. It is a high sensitive fluorometric assay used to measure protein, DNA and RNA which can give results in a short time. Even at low concentrations, the fluorescent dyes used in these tests give signals only when bound to specific target molecules, reducing the

effects of impurities and enabling the assay more sensitive compared to conventional Bradford Assays (Thermo Fisher Scientific Inc., 2014). After the calibration of device with proper standard solutions, the required reagents were added and samples were incubated at rt and measurement was done. Protein concentration results of different cattle breeds' milk are represented in Table 3.

Table 3

Protein concentration of different cattle breeds' milk.

Samples	**Protein concentration (mg/mL)**
Jersey	13.2 mg/mL
Holstein	13.8 mg/mL
Brown-Swiss	15.7 mg/mL
Guernsey	8.57mg/mL

Each sample's protein content were tested in dublicates. Firstly the concentration of samples were measured and the samples were out of range. To determine their concentrations all samples were diluted in 1:10 ratio and were measured respectively. After an incubation they were measured and the concentration of each was multiplied by the dilution factor. Based on the results; the protein concentration was higher for Brown-Swiss cattle compared to other breeds. The same results also was observed at the SDS-PAGE image, where the bands of Brown Swiss were denser than others. Additionally, several investigations revealed similar findings (Franzoi et al., 2019; Lim et al., 2020).

4.4. Enzymatic Deglycosylation of Milk Glycoproteins by PNGase F Enzyme

Deglycosylation of glycoproteins require enzyme to entirely remove the carbohydrate residues. The Peptidyl *N*-glycosidase F (PNGase F) enzyme is the commonly prefered *N*-linked deglycosylation enzyme at glycoanalytical processes for *N*-glycan removal (Vilaj et al., 2021). *N*-linked glycans were cut using a recombinantly produced PNGase F enzyme in BAP project (FHD-2021-3491).

4.4.1. Induction of Protein Expression and Purification of Enzyme

To induce the expression of protein from the recombinantly cloned gene, firstly into LB medium containing 33 µg/mL kanamycin was inoculated from 1% stock prepared from successful transformants and the cell density was achieved to reach OD_{600}nm: 0.5-0.6 under conditions of 37°C, 160 rpm. For the induction of protein expression was added having concentration of 0.1%. L-rhamnose. Following the incubation at 24°C all the night, induced cultures were centrifuged, and the obtained pellets were used for lysis procedure. Aim of the lysis process is to destroy the cell membrane to release protein from induced cells. For this, were used lysis buffer and as a second step was applied sonication to homogenize the cells. After sonication was performed centrifugation to clearly obtain the supernatant of lysed cells eliminated from cell debris.

The purification step was carried out by using a Batch method. At first step both in supernatant and resin was added equilibration buffer to verify that the proteins tagged with 6xHis bind efficiently with the resin. They were combined in single falcon tube and they incubated in order the proteins bind tightly to resin. While the 6xHis-tagged proteins attached to Ni resin, the other proteins were passed the system easily , as well. The solution including bound proteins on resin was washed with wash buffer to eliminate from undesirable impurities by applying repeated centrifugations. Subsequently, with the addition of elution buffer, proteins bound to resin were eluted and they were collected in a sterile falcon tube. For the concentration were used an Amicon tube (10-kDa cut-off).

The recombinantly produced enzyme purity was tested by SDS-PAGE gel electrophoresis. PNGase F enzyme is secreted from bacterium *Elizabethkingia miricola* ATCC 33958 has approximately 35 kDa molecular weight (Plummer et al., 1984). The amplified gene with N-His SUMO tag cause increase on the molecular weight , which is approximately 47 kDa (Figure 10).

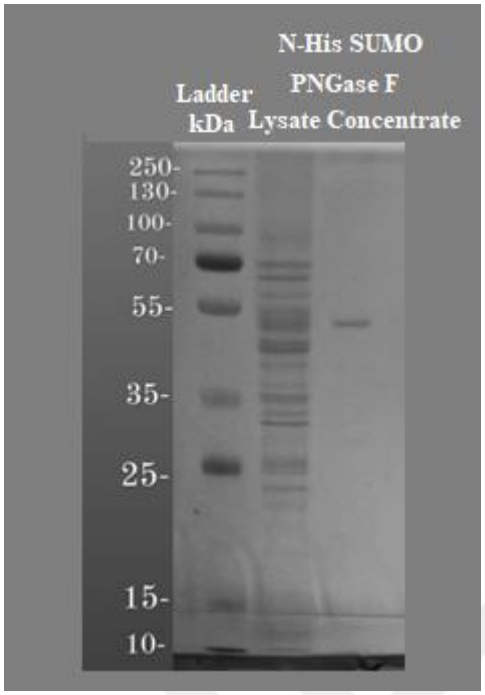

Figure 10. Analysis of purified N-His SUMO tagged PNGase F enzyme by SDS-PAGE.

4.4.2. Enzymatic Reaction for *N*-Glycan Release

The glycoprotein concentrations were calculated based on the PNGase F kit. It states that if the glycoprotein is in the range of 1-20 μg, the volume to be used is 10 μL. Since was required samples for SDS-PAGE, the total volume was increased to 50 μL which is out of range. The amount of glycoprotein to be used for the enzymatic reaction was calculated based on the Qubit result of each sample. Also, the volume of enzyme to be used for the reaction in kit is recommended to be 1μL, but for this reaction was used recombinantly produced enzyme. As a result, the volume of the enzyme for the denaturation was increased, also the incubation time was extended. Likewise, the volume of buffers used for the reaction was increased because the used glycoproteins was out of range. The volumes for the reaction were shown in Table 4.

Table 4

Reagents and volumes for the enzymatic reaction.

Tube Name	Jersey Control	Jersey with enzyme	Holstein Control	Holstein with enzyme	Brown-Swiss Control	Brown-Swiss with enzyme	Guernsey Control	Guernsey with enzyme
Glycoprotein	32 µL	32 µL	30 µL	30 µL	26 µL	26 µL	30 µL	30 µL
Glycoprotein Denaturing Buffer (10X)	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL	3 µL
dH_2O	3 µL	3 µL	5 µL	5 µL	9 µL	9 µL	5 µL	5 µL
Glycobuffer 2 (10X)	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL
10% NP-40	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL	4 µL
dH_2O	4 µL	-	4 µL	-	4 µL	-	4 µL	-
PNGase F	-	4 µL	-	4 µL	-	4 µL	-	4 µL
Total Volume	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**	**50 µL**

After incubation 20 µL from each sample was used for SDS-PAGE analysis to confirm the glycan separation from glycoproteins (Figure 11).

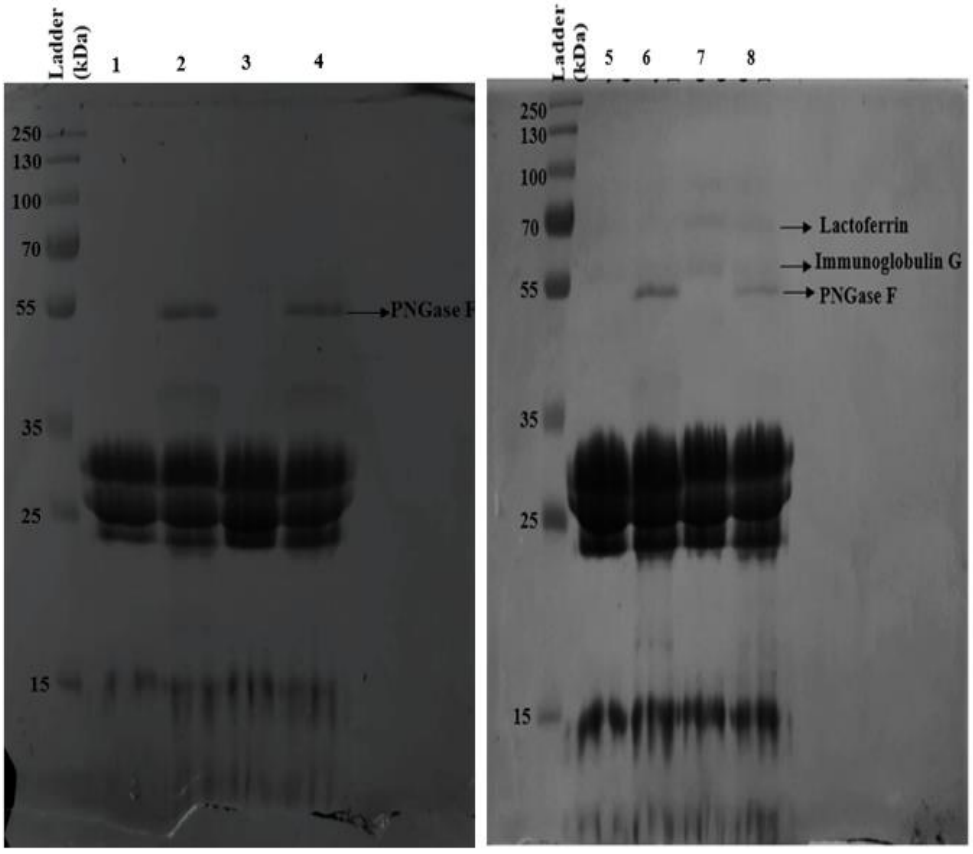

Figure 11. SDS-PAGE analysis of the glycoprotein deglycosylation by N-His SUMO tagged PNGase F enzyme. The enzymatic deglycosylation of glycoproteins at Figure a was not observed well, only the enzyme band exists at the expected kDa. Lane: 1 Holstein control. Lane: 2 Holstein treated with PNGase F. Lane: 3 Guernsey control. Lane: 4 Guernsey treated with PNGase F. At Figure b the deglycosylated glycoproteins can be seen. Lane: 5 Jersey control. Lane: 6 Jersey treated with PNGase F. Lane: 7 Brown Swiss control. Lane: 8 Brown Swiss treated with PNGase F.

4.5. Determination of Total Carbohydrate Content

For the quantification of released glycans was used Phenol Sulfuric Acid Assay (Figure 12). This method is very basic and timesaver for the detection of all carbohydrates using a standard curve of glucose. The main idea behind of this methodology is that; in acidic environment the glucose is dehydrated to the derivatives of furfural. This gives reaction with phenol and forms yellow, brown-coloured products (Figure 13).

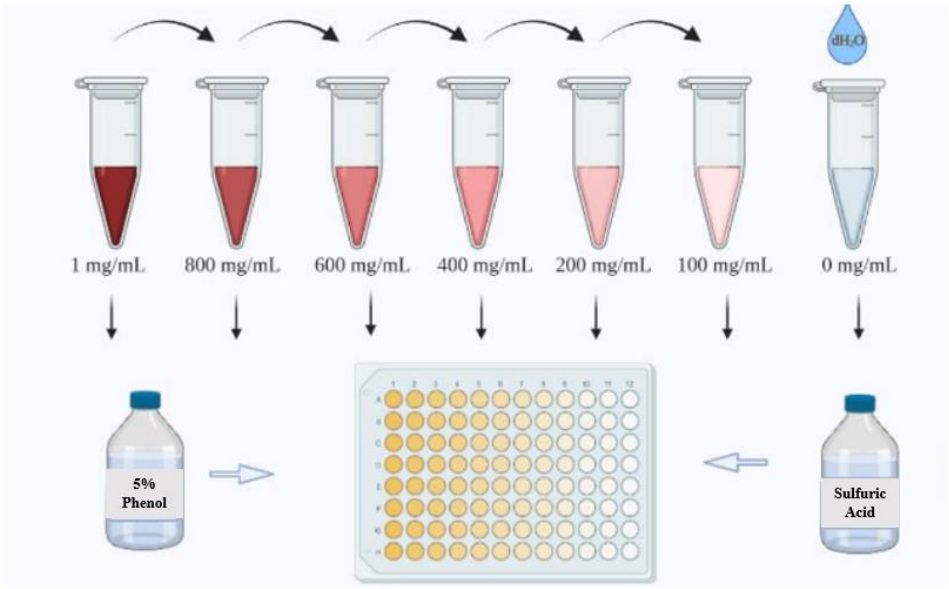

Figure 12. Preparation of standards and samples for Phenol Sulfuric Acid assay (Created by BioRender.com).

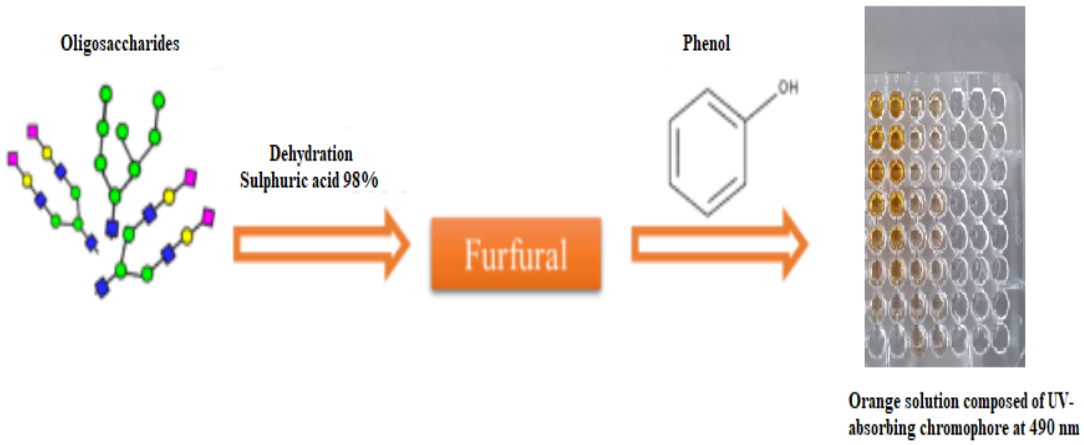

Figure 13. Schematic representation of Phenol Sulfuric Acid assay.

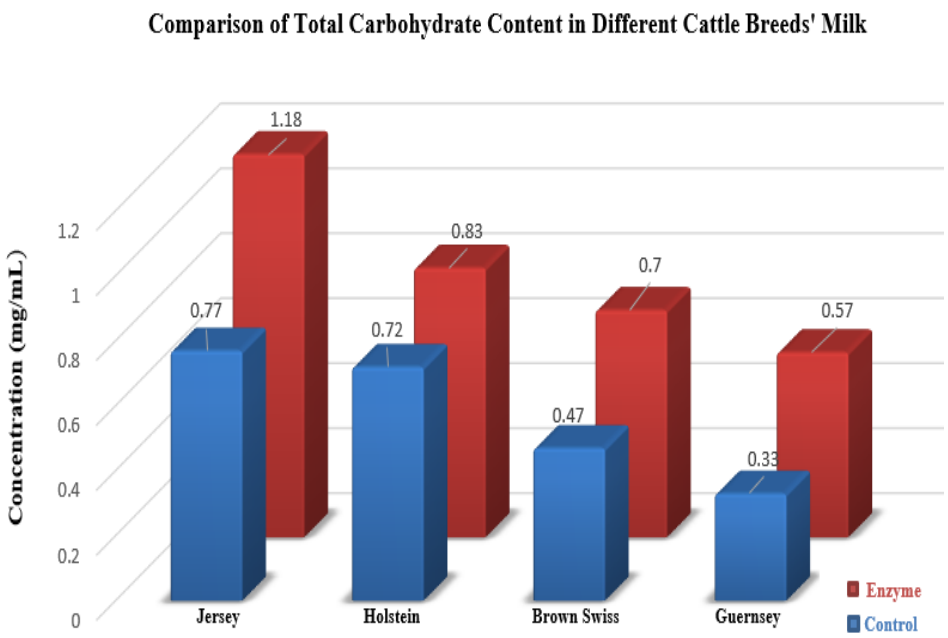

Figure 14. Comparison of carbohydrate content at different cattle breeds' milk. Control groups are represented with blue bars, while samples treated with PNGase F enzyme are represented with red bars.

The representative comparison of the carbohydrate content shown at Figure 14. As a result, the more release of glycan between breeds' milk observed to be in Jersey, which is 0.41 mg/mL, at Guernsey 0.24 mg/mL, at Brown Swiss 0.23 mg/mL and at Holstein 0.11 mg/mL respectively.

4.6. Detection of Lactoferrin Concentration in Milk of Various Cattle Breeds

Detection of lactoferrin concentration in milk of various cattle breeds was achieved by using a Sandwich ELISA assay, which quantifies the concentration of antigen in unknown sample. At sandwich model recognition of target antigen accomplished via anchoring by two antibodies which identifies different epitopes (Sakamoto et al., 2018). The pros of this model is its specificity, sensitivity and high and fast detection in an unknown sample the concentration of antigen (Shah and Maghsoudlou, 2016). At this assay samples and the standards were added in the wells, where each of them is pre-coated with unique lactoferrin

capture antibody, and the target antigens interact with specific antibody. The unbounds were washed down. After that was added detection antibody with biotin conjugation which attached to the captured antigen. Unattached detection antibody was washed. In consequence, into the well was added an Avidin-Horseradish Peroxidase (HRP) in order to attach to the biotin. The unattached HRP conjugate was removed. Onto this was added the TMB substrate which interacts with the HRP enzyme and leads to the color formation. Adding the sulfuric acid stop solution reaction was finished. The lactoferrin concentration of each breed was determined according to the plotted standard curve (Figure 6). The 1:1000 dilution factor was well fitted. According to the results the lactoferrin concentration of Holstein was 0.19 mg/mL, of Jersey 0.26 mg/mL, of Brown Swiss 0.28 mg/mL and Guernsey 0.18 mg/mL (Figure 15). The amount of lactoferrin found here are consistent with earlier findings (Oluk and Karaca, 2016; Recio et al., 2009).

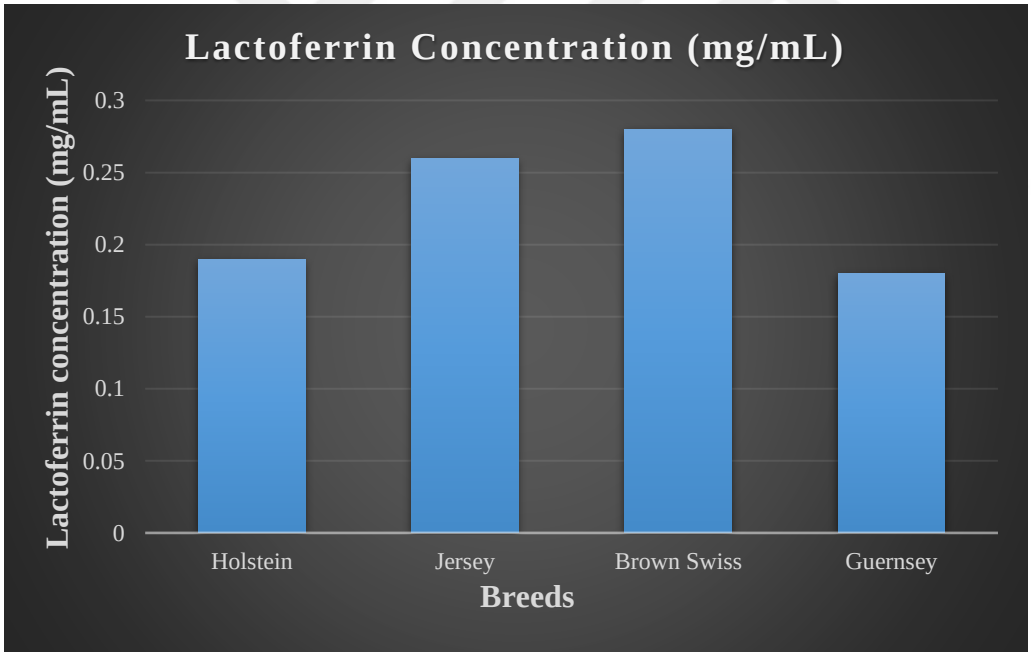

Figure 15. Lactoferrin content in each breeds' milk.

4.7. Detection of Immunoglobulin G (IgG) Concentration

The amount of IgG in milk samples was determined by using a competitive ELISA assay. At this assay all wells were pre-coated with a specific target capture antibody. Firstly,

the samples were added in each well as well as a fixed quantity of Horseradish Peroxidase (HRP)-conjugated detection antibody. Antigens and the free antigens found in the standards and the samples attached to the plate are in competition to bind on the detection antibody. The bound HRP-conjugated detection antibody and all were washed away. Onto them were added the TMB substrate which gives reaction with the HRP enzyme leading to color formation. The reaction was ended by additing sulfuric acid stop solution, where formation of color ends up. At competitive ELISA assay as the antigen concentration in sample is high, the change at optical density and colour formation is low. The IgG concentration of each sample was determined. The IgG concentration of Jersey found to be 0.35 mg/mL, of Holstein 0.42 mg/mL, of Guernsey 0.37 mg/mL and the concentration of Brown Swiss was 0.39 mg/mL. Based on the results was reported differences at the concentrations between breeds (Figure 16). At previous studies the milk IgG amount was 200-500 μg/mL, The concentration of IgG varies as the technique of measurement changes (Heck et al., 2009; Ulfman et al., 2018).

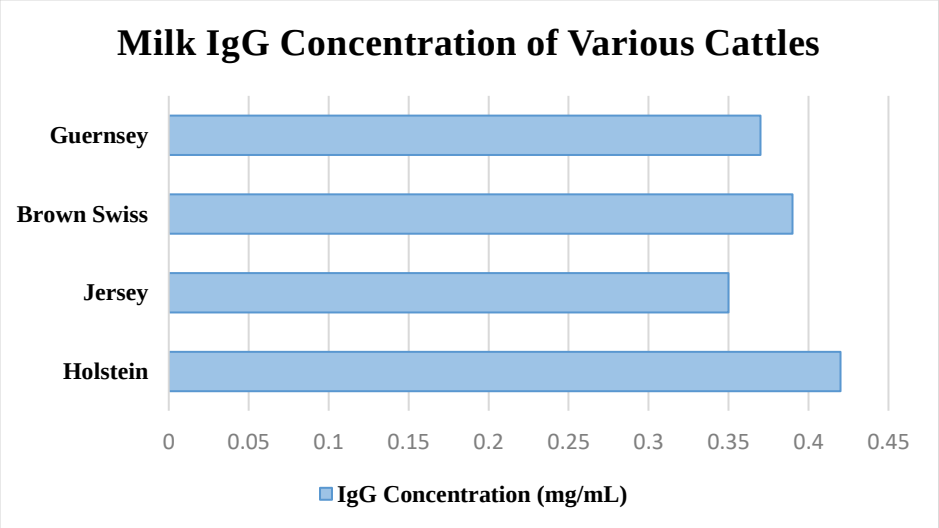

Figure 16. Presence of IgG's in milk of different breeds.

4.8 Characterization of Milk Glycans by MALDI-MS

Here there on Figure 17 were presented the MALDI-MS spectra of released *N*-glycans from milk samples of different breeds.

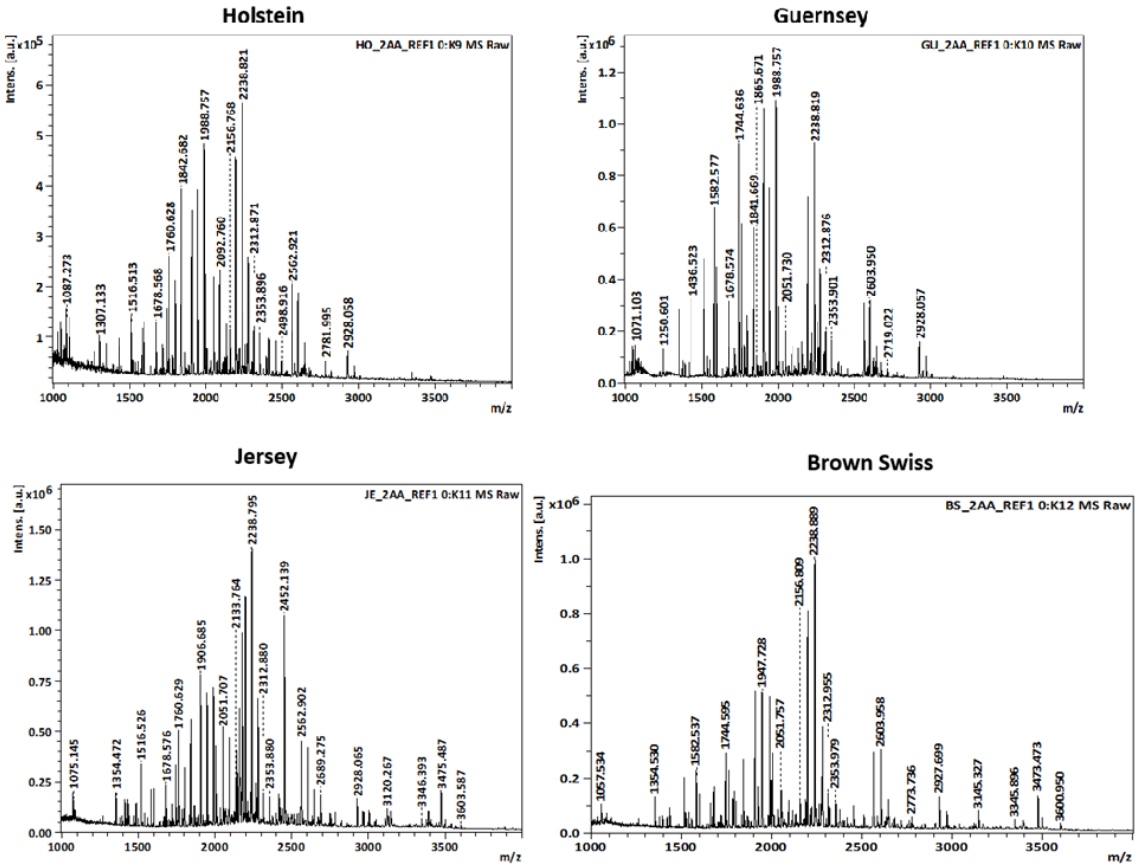

Figure 17. MALDI-MS spectra of released *N*-glycans from milk samples.

According to the results were observed different *N*-glycan compositions at each breed with different relative abundances. At milk of Brown Swiss were detected 45 different *N*-glycan structures, at Holstein's milk 39 *N*-glycan structures, at Jersey's milk 42 *N*-glycan structures and at Guernsey's milk 53 different *N*-glycan structures.

Table 5

N-glycan structures of Brown Swiss milk.

Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]	Int.	Δ m/z [ppm]	Score	IntCov. [%]	FragCov. [%]	Rt [min]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ MH+ [ppm]
1	Hex5HexNAc2-AA	1354.5301	-1	1354.4789	1355.4867	0.0512	118549	37.82					1356.5452	1356.4940	0.0512	37.77
2	Hex3HexNAc3dHex1-AA	1379.5450	-1	1379.5105	1380.5184	0.0345	56916	25.00					1381.5601	1381.5257	0.0345	24.96
3	Hex4HexNAc3-AA	1395.4697	-1	1395.5055	1396.5133	-0.0358	55694	-25.63					1397.4848	1397.5206	-0.0358	-25.59
4	Hex3HexNAc4-AA	1436.5259	-1	1436.5320	1437.5398	-0.0061	83375	-4.25					1438.5410	1438.5471	-0.0061	-4.24
5	Hex3HexNAc4S1-AA	1516.4905	-1	1516.4888	1517.4967	0.0017	187813	1.12					1518.5056	1518.5039	0.0017	1.12
6	Hex4HexNAc3dHex1-AA	1541.5167	-1	1541.5634	1542.5712	-0.0467	69594	-30.28					1543.5318	1543.5785	-0.0467	-30.25
7	Hex5HexNAc3-AA	1557.5019	-1	1557.5583	1558.5661	-0.0563	45459	-36.18					1559.5170	1559.5734	-0.0563	-36.13
8	Hex3HexNAc4dHex1-AA	1582.5371	-1	1582.5899	1583.5977	-0.0529	206692	-33.41					1584.5522	1584.6050	-0.0529	-33.37
9	Hex4HexNAc4-AA	1598.5300	-1	1598.5848	1599.5927	-0.0549	134725	-34.31					1600.5451	1600.5999	-0.0549	-34.27
10	Hex4HexNAc4S1-AA	1678.5276	-1	1678.5417	1679.5495	-0.0140	150595	-8.36					1680.5427	1680.5568	-0.0140	-8.35
11	Hex3HexNAc5S1-AA	1719.5491	-1	1719.5682	1720.5760	-0.0191	61638	-11.09					1721.5642	1721.5833	-0.0191	-11.08
12	Hex4HexNAc4dHex1-AA	1744.5952	-1	1744.6427	1745.6506	-0.0476	259035	-27.27					1746.6103	1746.6578	-0.0476	-27.23
13	Hex5HexNAc4-AA	1760.5930	-1	1760.6377	1761.6455	-0.0446	205611	-25.34					1762.6081	1762.6528	-0.0446	-25.31
14	Hex3HexNAc5dHex1-AA	1785.6323	-1	1785.6693	1786.6771	-0.0370	112487	-20.71					1787.6474	1787.6844	-0.0370	-20.69
15	Hex4HexNAc5-AA	1801.6274	-1	1801.6642	1802.6720	-0.0368	101724	-20.44					1803.6425	1803.6793	-0.0368	-20.41
16	Hex4HexNAc3NeuAc1dHex1-AA	1832.6139	-1	1832.6588	1833.6666	-0.0449	56764	-24.49					1834.6290	1834.6739	-0.0449	-24.46
17	Hex8HexNAc2-AA	1840.6181	-1	1840.6374	1841.6452	-0.0193	246465	-10.50					1842.6332	1842.6525	-0.0193	-10.49
18	Hex3HexNAc6-AA	1842.6690	-1	1842.6908	1843.6986	-0.0217	106322	-11.79					1844.6841	1844.7059	-0.0217	-11.77
19	Hex5HexNAc3NeuAc1-AA	1848.5715	-1	1848.6537	1849.6615	-0.0822	47579	-44.47					1850.5866	1850.6688	-0.0822	-44.42
20	Hex6HexNAc3dHex1-AA	1865.6484	-1	1865.6690	1866.6768	-0.0206	59311	-11.03					1867.6636	1867.6841	-0.0206	-11.01
21	Hex5HexNAc4dHex1-AA	1906.6875	-1	1906.6956	1907.7034	-0.0081	458565	-4.25					1908.7026	1908.7107	-0.0081	-4.24
22	Hex4HexNAc5dHex1-AA	1947.7284	-1	1947.7221	1948.7299	0.0063	476191	3.21					1949.7435	1949.7372	0.0063	3.21
23	Hex3HexNAc6dHex1-AA	1988.7679	-1	1988.7487	1989.7565	0.0192	460796	9.67					1990.7830	1990.7638	0.0192	9.66
24	Hex9HexNAc2-AA	2002.7136	-1	2002.6902	2003.6980	0.0234	264657	11.71					2004.7288	2004.7053	0.0234	11.69
25	Hex6HexNAc3NeuAc1-AA	2010.7114	-1	2010.7065	2011.7144	0.0048	54513	2.41					2012.7265	2012.7216	0.0048	2.41
26	Hex4HexNAc4NeuAc1dHex1-AA	2035.7539	-1	2035.7382	2036.7460	0.0158	76456	7.76					2037.7691	2037.7533	0.0158	7.75
27	Hex5HexNAc4NeuAc1-AA	2051.7569	-1	2051.7331	2052.7409	0.0238	143432	11.60					2053.7720	2053.7482	0.0238	11.59
28	Hex4HexNAc5NeuAc1-AA	2092.7977	-1	2092.7596	2093.7675	0.0381	106767	18.20					2094.8128	2094.7747	0.0381	18.19
29	Hex5HexNAc5dHex1-AA	2109.8212	-1	2109.7749	2110.7828	0.0462	41581	21.91					2111.8363	2111.7900	0.0462	21.89
30	Hex6HexNAc3NeuAc1dHex1-AA	2156.8094	-1	2156.7644	2157.7723	0.0450	112254	20.85					2158.8245	2158.7795	0.0450	20.83
31	Hex5HexNAc4NeuAc1dHex1-AA	2197.8541	-1	2197.7910	2198.7988	0.0631	762108	28.70					2199.8692	2199.8061	0.0631	28.67
32	Hex6HexNAc4NeuAc1-AA	2213.8414	-1	2213.7859	2214.7937	0.0555	55779	25.06					2215.8565	2215.8010	0.0555	25.04
33	Hex4HexNAc5NeuAc1dHex1-AA	2238.8893	-1	2238.8175	2239.8254	0.0717	994194	32.03					2240.9044	2240.8326	0.0717	32.00
34	Hex5HexNAc5NeuAc1-AA	2254.8780	-1	2254.8124	2255.8203	0.0655	40791	29.07					2256.8931	2256.8275	0.0655	29.04
35	Hex6HexNAc5dHex1-AA	2271.9148	-1	2271.8278	2272.8356	0.0870	104154	38.31					2273.9299	2273.8429	0.0870	38.27
36	Hex3HexNAc6NeuAc1dHex1-AA	2279.9205	-1	2279.8441	2280.8519	0.0764	371795	33.52					2281.9356	2281.8592	0.0764	33.50
37	Hex5HexNAc6dHex1-AA	2312.9552	-1	2312.8543	2313.8621	0.1009	146279	43.64					2314.9703	2314.8694	0.1009	43.60
38	Hex4HexNAc7dHex1-AA	2353.9787	-1	2353.8809	2354.8887	0.0978	106693	41.55					2355.9938	2355.8960	0.0978	41.51
39	Hex3HexNAc8dHex1-AA	2394.9940	-1	2394.9074	2395.9152	0.0866	41579	36.14					2397.0091	2396.9225	0.0866	36.11
40	Hex5HexNAc5NeuAc1dHex1-AA	2400.9339	-1	2400.8704	2401.8782	0.0636	37260	26.48					2402.9490	2402.8855	0.0636	26.46
41	Hex6HexNAc5NeuAc1-AA	2416.9366	-1	2416.8653	2417.8731	0.0713	51059	29.51					2418.9517	2418.8804	0.0713	29.49
42	Hex3HexNAc9-AA	2451.8493	-1	2451.9289	2452.9367	-0.0795	72283	-32.44					2453.8644	2453.9440	-0.0795	-32.41
43	Hex6HexNAc5NeuAc1dHex1-AA	2562.9526	-1	2562.9232	2563.9310	0.0295	290627	11.50					2564.9677	2564.9383	0.0295	11.49
44	Hex7HexNAc6dHex1-AA	2636.9767	-1	2636.9600	2637.9678	0.0167	53455	6.34					2638.9918	2638.9751	0.0167	6.34
45	Hex6HexNAc7dHex1-AA	2677.9537	-1	2677.9865	2678.9943	-0.0328	46068	-12.26					2679.9688	2680.0016	-0.0328	-12.26

Table 6

N-glycan structures of Guernsey milk.

Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]	Int.	Δ m/z [ppm]	Score	IntCov. [%]	FragCov. [%]	Rt [min]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ MH+ [ppm]
1	Hex5HexNAc2-AA	1354.4788	-1	1354.4789	1355.4867	-0.0001	265086	-0.07					1356.4939	1356.4940	-0.0001	-0.07
2	Hex3HexNAc3dHex1-AA	1379.5094	-1	1379.5105	1380.5184	-0.0011	81779	-0.82					1381.5245	1381.5257	-0.0011	-0.82
3	Hex4HexNAc3-AA	1395.4897	-1	1395.5055	1396.5133	-0.0158	65830	-11.31					1397.5048	1397.5206	-0.0158	-11.30
4	Hex3HexNAc4-AA	1436.5231	-1	1436.5320	1437.5398	-0.0089	291571	-6.21					1438.5382	1438.5471	-0.0089	-6.20
5	Hex6HexNAc2-AA	1516.5180	-1	1516.5317	1517.5396	-0.0138	441509	-9.08					1518.5331	1518.5468	-0.0138	-9.07
6	Hex4HexNAc3dHex1-AA	1541.5487	-1	1541.5634	1542.5712	-0.0147	94613	-9.53					1543.5638	1543.5785	-0.0147	-9.52
7	Hex5HexNAc3-AA	1557.5442	-1	1557.5583	1558.5661	-0.0140	83857	-9.02					1559.5593	1559.5734	-0.0140	-9.01
8	Hex3HexNAc4dHex1-AA	1582.5768	-1	1582.5899	1583.5977	-0.0131	622620	-8.30					1584.5919	1584.6050	-0.0131	-8.29
9	Hex4HexNAc4-AA	1598.5721	-1	1598.5848	1599.5927	-0.0127	411411	-7.97					1600.5872	1600.5999	-0.0127	-7.96
10	Hex3HexNAc5-AA	1639.5946	-1	1639.6114	1640.6192	-0.0167	48725	-10.21					1641.6097	1641.6265	-0.0167	-10.20
11	Hex7HexNAc2-AA	1678.5741	-1	1678.5846	1679.5924	-0.0104	282058	-6.21					1680.5892	1680.5997	-0.0104	-6.20
12	Hex5HexNAc3dHex1-AA	1703.6070	-1	1703.6162	1704.6240	-0.0092	51218	-5.42					1705.6221	1705.6313	-0.0092	-5.41
13	Hex6HexNAc3-AA	1719.6022	-1	1719.6111	1720.6189	-0.0090	121054	-5.21					1721.6173	1721.6262	-0.0090	-5.20
14	Hex3HexNAc4NeuAc1-AA	1727.6076	-1	1727.6274	1728.6353	-0.0198	61446	-11.49					1729.6227	1729.6425	-0.0198	-11.48
15	Hex4HexNAc4dHex1-AA	1744.6364	-1	1744.6427	1745.6506	-0.0063	861887	-3.62					1746.6515	1746.6578	-0.0063	-3.62
16	Hex5HexNAc4-AA	1760.6323	-1	1760.6377	1761.6455	-0.0053	556165	-3.03					1762.6474	1762.6528	-0.0053	-3.03
17	Hex3HexNAc5dHex1-AA	1785.6647	-1	1785.6693	1786.6771	-0.0046	136755	-2.59					1787.6798	1787.6844	-0.0046	-2.58
18	Hex4HexNAc5-AA	1801.6607	-1	1801.6642	1802.6720	-0.0035	231587	-1.94					1803.6758	1803.6793	-0.0035	-1.94
19	Hex4HexNAc3NeuAc1dHex1-AA	1832.6420	-1	1832.6588	1833.6666	-0.0168	53244	-9.14					1834.6571	1834.6739	-0.0168	-9.13
20	Hex6HexNAc3dHex1-AA	1865.6706	-1	1865.6690	1866.6768	0.0016	94565	0.87					1867.6857	1867.6841	0.0016	0.86
21	Hex4HexNAc4NeuAc1-AA	1889.6743	-1	1889.6803	1890.6881	-0.0060	63998	-3.16					1891.6894	1891.6954	-0.0060	-3.16
22	Hex5HexNAc4dHex1-AA	1906.6993	-1	1906.6956	1907.7034	0.0037	958623	1.96					1908.7144	1908.7107	0.0037	1.96
23	Hex3HexNAc6S1-AA	1922.6637	-1	1922.6476	1923.6554	0.0161	75360	8.39					1924.6788	1924.6627	0.0161	8.38
24	Hex4HexNAc5dHex1-AA	1947.7276	-1	1947.7221	1948.7299	0.0054	711014	2.80					1949.7427	1949.7372	0.0054	2.79
25	Hex3HexNAc6dHex1-AA	1988.7569	-1	1988.7487	1989.7565	0.0083	1037646	4.16					1990.7720	1990.7638	0.0083	4.16
26	Hex9HexNAc2-AA	2002.6989	-1	2002.6902	2003.6980	0.0087	271442	4.36					2004.7140	2004.7053	0.0087	4.36
27	Hex6HexNAc3NeuAc1-AA	2010.6999	-1	2010.7065	2011.7144	-0.0066	72019	-3.29					2012.7150	2012.7216	-0.0066	-3.29
28	Hex4HexNAc4NeuAc1dHex1-AA	2035.7305	-1	2035.7382	2036.7460	-0.0077	75372	-3.76					2037.7456	2037.7533	-0.0077	-3.76
29	Hex5HexNAc4NeuAc1-AA	2051.7305	-1	2051.7331	2052.7409	-0.0026	187547	-1.27					2053.7456	2053.7482	-0.0026	-1.27
30	Hex5HexNAc4NeuGc1-AA	2067.7342	-1	2067.7280	2068.7358	0.0063	60242	3.03					2069.7431	2069.7431	0.0063	3.02
31	Hex4HexNAc5NeuAc1-AA	2092.7630	-1	2092.7596	2093.7675	0.0034	139541	1.61					2094.7781	2094.7747	0.0034	1.61
32	Hex5HexNAc5dHex1-AA	2109.7799	-1	2109.7749	2110.7828	0.0050	63570	2.37					2111.7950	2111.7900	0.0050	2.36
33	Hex6HexNAc5-AA	2125.7754	-1	2125.7699	2126.7777	0.0056	49338	2.62					2127.7905	2127.7850	0.0056	2.62
34	Hex6HexNAc3NeuAc1dHex1-AA	2156.7633	-1	2156.7644	2157.7723	-0.0011	144572	-0.51					2158.7784	2158.7795	-0.0011	-0.51
35	Hex10HexNAc2-AA	2164.7614	-1	2164.7430	2165.7509	0.0183	50673	8.48					2166.7765	2166.7581	0.0183	8.47
36	Hex5HexNAc4NeuAc1dHex1-AA	2197.7912	-1	2197.7910	2198.7988	0.0002	720316	0.10					2199.8061	2199.8061	0.0002	0.10
37	Hex6HexNAc4NeuAc1-AA	2213.7884	-1	2213.7859	2214.7937	0.0025	85903	1.13					2215.8035	2215.8010	0.0025	1.13
38	Hex4HexNAc5NeuAc1dHex1-AA	2238.8191	-1	2238.8175	2239.8254	0.0015	911442	0.69					2240.8342	2240.8326	0.0015	0.69
39	Hex5HexNAc5NeuAc1-AA	2254.8169	-1	2254.8124	2255.8203	0.0045	49940	1.99					2256.8320	2256.8275	0.0045	1.99
40	Hex5HexNAc4KDN2-AA	2260.8184	-1	2260.7754	2261.7832	0.0430	233964	19.03					2262.8335	2262.7905	0.0430	19.01
41	Hex6HexNAc5dHex1-AA	2271.8456	-1	2271.8278	2272.8356	0.0179	134132	7.86					2273.8607	2273.8429	0.0179	7.85
42	Hex3HexNAc6NeuAc1dHex1-AA	2279.8454	-1	2279.8441	2280.8519	0.0013	424354	0.56					2281.8605	2281.8592	0.0013	0.56
43	Hex5HexNAc6dHex1-AA	2312.8761	-1	2312.8543	2313.8621	0.0218	199174	9.42					2314.8912	2314.8694	0.0218	9.41
44	Hex4HexNAc7dHex1-AA	2353.9011	-1	2353.8809	2354.8887	0.0202	164943	8.58					2355.9162	2355.8960	0.0202	8.57
45	Hex6HexNAc4NeuGc1dHex1-AA	2375.7917	-1	2375.8387	2376.8465	-0.0471	43522	-19.81					2377.8068	2377.8538	-0.0471	-19.79
46	Hex3HexNAc8dHex1-AA	2394.9225	-1	2394.9074	2395.9152	0.0151	79278	6.30					2396.9376	2396.9225	0.0151	6.30
47	Hex6HexNAc5NeuAc1-AA	2416.8655	-1	2416.8653	2417.8731	0.0002	60328	0.09					2418.8806	2418.8804	0.0002	0.09
48	Hex6HexNAc5NeuAc1dHex1-AA	2562.9185	-1	2562.9232	2563.9310	-0.0047	293276	-1.83					2564.9336	2564.9383	-0.0047	-1.82
49	Hex7HexNAc6dHex1-AA	2636.9773	-1	2636.9600	2637.9678	0.0174	73236	6.59					2638.9924	2638.9751	0.0174	6.59
50	Hex6HexNAc5Neu1dHex2-AA	2666.9667	-1	2666.9705	2667.9783	-0.0038	41243	-1.43					2668.9818	2668.9856	-0.0038	-1.43
51	Hex6HexNAc7dHex1-AA	2678.0033	-1	2677.9865	2678.9943	0.0168	75063	6.26					2680.0184	2680.0016	0.0168	6.25
52	Hex5HexNAc8dHex1-AA	2719.0216	-1	2719.0131	2720.0209	0.0085	42336	3.13					2721.0367	2721.0282	0.0085	3.13
53	Hex7HexNAc6NeuAc1dHex1-AA	2928.0566	-1	2928.0554	2929.0632	0.0013	145978	0.43					2930.0717	2930.0705	0.0013	0.43

Table 7

N-glycan structures of Holstein milk.

Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]	Int.	Δ m/z [ppm]	Score	IntCov. [%]	FragCov. [%]	Rt [min]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ MH+ [ppm]
1	Hex5HexNAc2-AA	1354.4806	-1	1354.4789	1355.4867	0.0017	78952	1.25					1356.4957	1356.4940	0.0017	1.25
2	Hex3HexNAc4-AA	1436.5164	-1	1436.5320	1437.5398	-0.0157	83578	-10.90					1438.5315	1438.5471	-0.0157	-10.88
3	Hex6HexNAc2-AA	1516.5130	-1	1516.5317	1517.5396	-0.0187	128892	-12.34					1518.5281	1518.5468	-0.0187	-12.33
4	Hex3HexNAc4dHex1-AA	1582.5719	-1	1582.5899	1583.5977	-0.0180	107806	-11.37					1584.5870	1584.6050	-0.0180	-11.35
5	Hex4HexNAc4-AA	1598.5657	-1	1598.5848	1599.5927	-0.0192	111842	-11.99					1600.5808	1600.5999	-0.0192	-11.97
6	Hex7HexNAc2-AA	1678.5686	-1	1678.5846	1679.5924	-0.0160	110999	-9.54					1680.5837	1680.5997	-0.0160	-9.53
7	Hex6HexNAc3-AA	1719.5995	-1	1719.6111	1720.6189	-0.0116	72694	-6.75					1721.6146	1721.6262	-0.0116	-6.75
8	Hex4HexNAc4dHex1-AA	1744.6333	-1	1744.6427	1745.6506	-0.0094	142471	-5.39					1746.6484	1746.6578	-0.0094	-5.39
9	Hex5HexNAc4-AA	1760.6284	-1	1760.6377	1761.6455	-0.0093	232728	-5.27					1762.6435	1762.6528	-0.0093	-5.26
10	Hex3HexNAc5dHex1-AA	1785.6562	-1	1785.6693	1786.6771	-0.0131	57436	-7.34					1787.6713	1787.6844	-0.0131	-7.33
11	Hex4HexNAc5-AA	1801.6586	-1	1801.6642	1802.6720	-0.0056	191920	-3.11					1803.6737	1803.6793	-0.0056	-3.11
12	Hex8HexNAc2-AA	1840.6350	-1	1840.6374	1841.6452	-0.0024	197375	-1.31					1842.6501	1842.6525	-0.0024	-1.31
13	Hex3HexNAc6-AA	1842.6823	-1	1842.6908	1843.6986	-0.0085	275602	-4.60					1844.6974	1844.7059	-0.0085	-4.59
14	Hex6HexNAc3dHex1-AA	1865.6693	-1	1865.6690	1866.6768	0.0002	63074	0.13					1867.6844	1867.6841	0.0002	0.13
15	Hex5HexNAc4dHex1-AA	1906.6982	-1	1906.6956	1907.7034	0.0026	315607	1.36					1908.7133	1908.7107	0.0026	1.36
16	Hex4HexNAc5dHex1-AA	1947.7278	-1	1947.7221	1948.7299	0.0057	351411	2.92					1949.7429	1949.7372	0.0057	2.91
17	Hex3HexNAc6dHex1-AA	1988.7570	-1	1988.7487	1989.7565	0.0083	455701	4.18					1990.7721	1990.7638	0.0083	4.17
18	Hex9HexNAc2-AA	2002.7007	-1	2002.6902	2003.6980	0.0105	213147	5.25					2004.7158	2004.7053	0.0105	5.24
19	Hex6HexNAc3NeuAc1-AA	2010.6983	-1	2010.7065	2011.7144	-0.0082	69127	-4.10					2012.7134	2012.7216	-0.0082	-4.10
20	Hex5HexNAc4NeuAc1-AA	2051.7311	-1	2051.7331	2052.7409	-0.0020	206918	-0.97					2053.7462	2053.7482	-0.0020	-0.97
21	Hex4HexNAc5NeuAc1-AA	2092.7604	-1	2092.7596	2093.7675	0.0008	224625	0.39					2094.7755	2094.7747	0.0008	0.39
22	Hex6HexNAc5-AA	2125.7822	-1	2125.7699	2126.7777	0.0123	53026	5.79					2127.7973	2127.7850	0.0123	5.79
23	Hex6HexNAc3NeuAc1dHex1-AA	2156.7675	-1	2156.7644	2157.7723	0.0031	104947	1.44					2158.7826	2158.7795	0.0031	1.44
24	Hex5HexNAc6-AA	2166.8038	-1	2166.7964	2167.8042	0.0074	67721	3.40					2168.8189	2168.8115	0.0074	3.40
25	Hex5HexNAc4NeuAc1dHex1-AA	2197.7930	-1	2197.7910	2198.7988	0.0020	451545	0.92					2199.8081	2199.8061	0.0020	0.92
26	Hex4HexNAc7-AA	2207.8313	-1	2207.8230	2208.8308	0.0084	60075	3.80					2209.8464	2209.8381	0.0084	3.80
27	Hex6HexNAc4NeuAc1-AA	2213.7837	-1	2213.7859	2214.7937	-0.0022	49891	-1.00					2215.7988	2215.8010	-0.0022	-1.00
28	Hex4HexNAc5NeuAc1dHex1-AA	2238.8209	-1	2238.8175	2239.8254	0.0033	551975	1.49					2240.8360	2240.8326	0.0033	1.49
29	Hex5HexNAc4KDN2-AA	2260.8247	-1	2260.7754	2261.7832	0.0493	84120	21.79					2262.8398	2262.7905	0.0493	21.77
30	Hex6HexNAc5dHex1-AA	2271.8422	-1	2271.8278	2272.8356	0.0144	88331	6.35					2273.8573	2273.8429	0.0144	6.34
31	Hex3HexNAc6NeuAc1dHex1-AA	2279.8467	-1	2279.8441	2280.8519	0.0026	252884	1.14					2281.8618	2281.8592	0.0026	1.14
32	Hex5HexNAc6dHex1-AA	2312.8709	-1	2312.8543	2313.8621	0.0166	114675	7.17					2314.8860	2314.8694	0.0166	7.17
33	Hex4HexNAc7dHex1-AA	2353.8958	-1	2353.8809	2354.8887	0.0150	103412	6.36					2355.9109	2355.8960	0.0150	6.36
34	Hex3HexNAc8dHex1-AA	2394.9147	-1	2394.9074	2395.9152	0.0073	58520	3.05					2396.9298	2396.9225	0.0073	3.05
35	Hex6HexNAc5NeuAc1-AA	2416.8632	-1	2416.8653	2417.8731	-0.0021	97326	-0.87					2418.8783	2418.8804	-0.0021	-0.87
36	Hex6HexNAc5NeuAc1dHex1-AA	2562.9211	-1	2562.9232	2563.9310	-0.0020	198414	-0.79					2564.9362	2564.9383	-0.0020	-0.79
37	Hex7HexNAc6dHex1-AA	2636.9677	-1	2636.9600	2637.9678	0.0078	41884	2.95					2638.9828	2638.9751	0.0078	2.95
38	Hex7HexNAc6NeuAc1-AA	2781.9947	-1	2781.9975	2783.0053	-0.0028	45745	-1.00					2784.0098	2784.0126	-0.0028	-1.00
39	Hex7HexNAc6NeuAc1dHex1-AA	2928.0580	-1	2928.0554	2929.0632	0.0027	66029	0.91					2930.0731	2930.0705	0.0027	0.91

Table 8

N-glycan structures of Jersey milk.

Row	Composition	m/z meas.	z	m/z calc.	Mr calc.	Δ MH+ [Da]	Int.	Δ m/z [ppm]	Score	IntCov. [%]	FragCov. [%]	Rt [min]	MH+ meas.	MH+ calc.	Δ m/z [Da]	Δ MH+ [ppm]
1	Hex5HexNAc2-AA	1354.4717	-1	1354.4789	1355.4867	-0.0073	176665	-5.36					1356.4868	1356.4940	-0.0073	-5.35
2	Hex3HexNAc4-AA	1436.5287	-1	1436.5320	1437.5398	-0.0033	128178	-2.30					1438.5438	1438.5471	-0.0033	-2.30
3	Hex6HexNAc2-AA	1516.5256	-1	1516.5317	1517.5396	-0.0061	313693	-4.05					1518.5407	1518.5468	-0.0061	-4.05
4	Hex4HexNAc3dHex1-AA	1541.5519	-1	1541.5634	1542.5712	-0.0115	83046	-7.46					1543.5670	1543.5785	-0.0115	-7.45
5	Hex3HexNAc4dHex1-AA	1582.5858	-1	1582.5899	1583.5977	-0.0042	196029	-2.63					1584.6009	1584.6050	-0.0042	-2.62
6	Hex4HexNAc4-AA	1598.5777	-1	1598.5848	1599.5927	-0.0071	202043	-4.44					1600.5928	1600.5999	-0.0071	-4.43
7	Hex7HexNAc2-AA	1678.5764	-1	1678.5846	1679.5924	-0.0082	209081	-4.88					1680.5915	1680.5997	-0.0082	-4.87
8	Hex6HexNAc3-AA	1719.6014	-1	1719.6111	1720.6189	-0.0097	123916	-5.64					1721.6165	1721.6262	-0.0097	-5.63
9	Hex4HexNAc4dHex1-AA	1744.6360	-1	1744.6427	1745.6506	-0.0067	305040	-3.87					1746.6511	1746.6578	-0.0067	-3.86
10	Hex5HexNAc4-AA	1760.6289	-1	1760.6377	1761.6455	-0.0088	433274	-4.98					1762.6440	1762.6528	-0.0088	-4.97
11	Hex3HexNAc5dHex1-AA	1785.6584	-1	1785.6693	1786.6771	-0.0109	102524	-6.10					1787.6735	1787.6844	-0.0109	-6.09
12	Hex4HexNAc5-AA	1801.6576	-1	1801.6642	1802.6720	-0.0066	280467	-3.67					1803.6727	1803.6793	-0.0066	-3.66
13	Hex4HexNAc3NeuAc1dHex1-AA	1832.6294	-1	1832.6588	1833.6666	-0.0294	73890	-16.06					1834.6445	1834.6739	-0.0294	-16.04
14	Hex8HexNAc2-AA	1840.6402	-1	1840.6374	1841.6452	0.0028	401721	1.51					1842.6553	1842.6525	0.0028	1.51
15	Hex3HexNAc6-AA	1842.6905	-1	1842.6908	1843.6986	-0.0003	356193	-0.14					1844.7056	1844.7059	-0.0003	-0.14
16	Hex5HexNAc3NeuAc1-AA	1848.6196	-1	1848.6537	1849.6615	-0.0341	68626	-18.46					1850.6347	1850.6688	-0.0341	-18.44
17	Hex6HexNAc3dHex1-AA	1865.6559	-1	1865.6690	1866.6768	-0.0131	112413	-7.03					1867.6710	1867.6841	-0.0131	-7.03
18	Hex4HexNAc4NeuAc1-AA	1889.6564	-1	1889.6803	1890.6881	-0.0239	74178	-12.65					1891.6715	1891.6954	-0.0239	-12.63
19	Hex5HexNAc4dHex1-AA	1906.6847	-1	1906.6956	1907.7034	-0.0108	699760	-5.69					1908.6998	1908.7107	-0.0108	-5.68
20	Hex3HexNAc6S1-AA	1922.6424	-1	1922.6476	1923.6554	-0.0052	74178	-2.68					1924.6575	1924.6627	-0.0052	-2.68
21	Hex4HexNAc5dHex1-AA	1947.7114	-1	1947.7221	1948.7299	-0.0108	628648	-5.52					1949.7265	1949.7372	-0.0108	-5.52
22	Hex3HexNAc6dHex1-AA	1988.7406	-1	1988.7487	1989.7565	-0.0080	673952	-4.04					1990.7557	1990.7638	-0.0080	-4.03
23	Hex9HexNAc2-AA	2002.6795	-1	2002.6902	2003.6980	-0.0108	389043	-5.37					2004.6946	2004.7053	-0.0108	-5.37
24	Hex6HexNAc3NeuAc1-AA	2010.6796	-1	2010.7065	2011.7144	-0.0269	152926	-13.37					2012.6947	2012.7216	-0.0269	-13.36
25	Hex4HexNAc4NeuAc1dHex1-AA	2035.7086	-1	2035.7382	2036.7460	-0.0295	104436	-14.51					2037.7237	2037.7533	-0.0295	-14.50
26	Hex5HexNAc4NeuAc1-AA	2051.7075	-1	2051.7331	2052.7409	-0.0256	480357	-12.47					2053.7226	2053.7482	-0.0256	-12.46
27	Hex4HexNAc5NeuAc1-AA	2092.7334	-1	2092.7596	2093.7675	-0.0262	447510	-12.53					2094.7485	2094.7747	-0.0262	-12.52
28	Hex6HexNAc5-AA	2125.7477	-1	2125.7699	2126.7777	-0.0222	85560	-10.43					2127.7628	2127.7850	-0.0222	-10.42
29	Hex5HexNAc4NeuAc1dHex1-AA	2197.7621	-1	2197.7910	2198.7988	-0.0289	1121280	-13.14					2199.7772	2199.8061	-0.0289	-13.13
30	Hex4HexNAc5NeuAc1dHex1-AA	2238.7945	-1	2238.8175	2239.8254	-0.0230	1311209	-10.28					2240.8096	2240.8326	-0.0230	-10.27
31	Hex5HexNAc4KDN2-AA	2260.7838	-1	2260.7754	2261.7832	0.0085	75820	3.74					2262.7989	2262.7905	0.0085	3.74
32	Hex6HexNAc5dHex1-AA	2271.8171	-1	2271.8278	2272.8356	-0.0107	145409	-4.71					2273.8322	2273.8429	-0.0107	-4.71
33	Hex3HexNAc6NeuAc1dHex1-AA	2279.8225	-1	2279.8441	2280.8519	-0.0215	645349	-9.45					2281.8376	2281.8592	-0.0215	-9.44
34	Hex5HexNAc6dHex1-AA	2312.8800	-1	2312.8543	2313.8621	0.0256	198991	11.09					2314.8951	2314.8694	0.0256	11.08
35	Hex4HexNAc7dHex1-AA	2353.8798	-1	2353.8809	2354.8887	-0.0011	154991	-0.46					2355.8949	2355.8960	-0.0011	-0.46
36	Hex5HexNAc4NeuGc2-AA	2374.7924	-1	2374.8183	2375.8261	-0.0260	64988	-10.93					2376.8075	2376.8334	-0.0260	-10.92
37	Hex3HexNAc8dHex1-AA	2394.9110	-1	2394.9074	2395.9152	0.0036	89252	1.50					2396.9261	2396.9225	0.0036	1.50
38	Hex6HexNAc5NeuAc1-AA	2416.8418	-1	2416.8653	2417.8731	-0.0235	181235	-9.70					2418.8569	2418.8804	-0.0235	-9.70
39	Hex6HexNAc5NeuAc1dHex1-AA	2562.9022	-1	2562.9232	2563.9310	-0.0210	406733	-8.19					2564.9173	2564.9383	-0.0210	-8.19
40	Hex7HexNAc6dHex1-AA	2636.9707	-1	2636.9600	2637.9678	0.0107	69481	4.07					2638.9858	2638.9751	0.0107	4.07
41	Hex7HexNAc6NeuAc1-AA	2781.9930	-1	2781.9975	2783.0053	-0.0045	87550	-1.62					2784.0081	2784.0126	-0.0045	-1.62
42	Hex7HexNAc6NeuAc1dHex1-AA	2928.0649	-1	2928.0554	2929.0632	0.0095	147211	3.24					2930.0800	2930.0705	0.0095	3.24

CHAPTER 5
RESULTS AND RECOMMENDATIONS

Within the scope of this thesis study was revealed the changes in protein, IgG and lactoferrin concentrations in milk of different breeds. As it is known there are many determinants affecting the composition of milk. These can be period of lactation, breed, the health status of cattle and the environmental and seasonal effects.

In this study considering the effect of breed, was examined how the breed can differs in terms of milk components. The milk proteins are vital for the satisfaction of nutritional needs of newborns. The most valuable component of milk is regarded to be the proteins. The reason for this is because milk proteins are increasingly recognized as having superior nutritional and functional qualities than those of other protein sources. For this reason is important for animal husbandry to raise cattle breeds with a great capacity to produce milk offering high protein content.

Regarding the composition of Holstein, Jersey, Brown Swiss and Guernsey's milk the content of protein referred to milk of Brown Swiss was found to be the richest compared to other breeds' milk. On the other hand, the milk of Holstein and Jersey showed insignificant differences in content of protein. As previously stated the properties of Brown Swiss, which are growing in long period having slow growth rate and reaching to maturity later, but living for a long time. They are considered to be the best breeds for protein power capacity and milk productivity.

Furthermore it is important to report that the Brown Swiss' milk in addition to the highest protein amount being compatible with ELISA results.displayed the higher concentration of lactoferrin, which presents biological features including anti-inflammatory and antimicrobial activities.

The high content of multifunctional protein IgG were observed to be at Holstein as well as indicating small difference between Brown Swiss. As it is known that the IgG levels alters throughtout the transition period, also it was observed to change between breeds. Detection of IgG and lactoferrin quantities was determined by using ELISA assay, being the most commonly and preferably method for the detection of components of plasma, serum and many other fluids even if they are found in low concentrations.

To study the glycan structures they have to be properly liberated from glycoproteins. As were previously referred, the glycans cut from glycoproteins by using enzymes and chemicals. But the use of chemicals have disadvantages, such as creating difficulties in further analysis. For this reason were used enzyme, the PNGase F which was produced from recombinantly cloned gene. The purification of N-His SUMO tagged enzyme was achieved by using Ni-NTA resin. Here there the use of tag is very crucial because the SUMO tag has the ability to meliorate the protein solubility and helps for protein purification.

After obtaining the enzyme, the deglycosylation process was performed, to release the glycans from the glycoproteins applying overnight incubation. In consequence these glycans were characterized by MALDI-MS analysis to understand which glycans are mostly found and to compare the glycan content between breeds. According to the results, were detected different *N*-glycan structures at each breed. It was considered that the varieties at glycan compositions can affect shaping the gut microbiota both on neonates and adults.

This information can be handled by dairy farms raising cattle breeds for the enhancement of milk characteristics, in order to get qualified products. Because the milk components such as the milk proteins, lactoferrin can be used in industrial areas as food additives and also can be used for therapeutic reasons.

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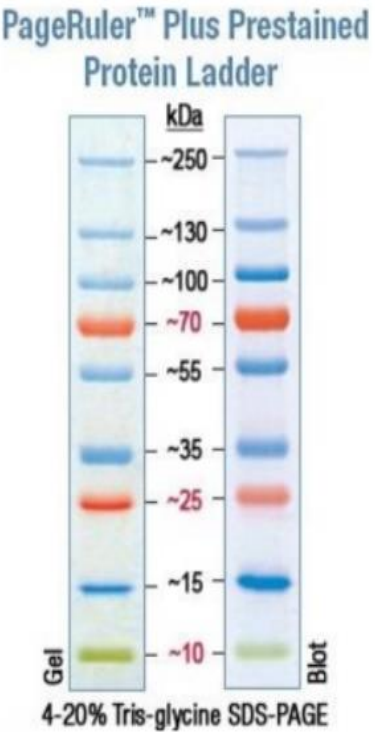
APPENDICES

APPENDIX 1

PROTEIN LADDER USED AS A MARKER



APPENDIX 2

BUFFERS AND INGREDIENTS USED DURING PROTEIN PURIFICATION

	Lysis Buffer (pH 8.0)	**Equilibration Buffer (pH 7.4)**	**Wash Buffer (pH 7.4)**	**Elution Buffer (pH 7.4)**
Ingredients				
Imidazole	1 mM	10 mM	25 mM	250 mM
Tris-HCl	50 mM	-	-	-
NaCl	200 mM	300 mM	300 mM	300 mM
NaH_2PO_4	-	20 mM	20 mM	20 mM
SDS	1%	-	-	-